CHORISMATE LYASE

AND

UBIQUINONE BIOSYNTHESIS

IN

ESCHERICHIA COLI K-12.

A THESIS

Submitted for the degree of

DOCTOR OF PHILOSOPHY

at the

AUSTRALIAN NATIONAL UNIVERSITY

1973.

PREFACE.

This thesis embodies the results of research carried out in the Department of Biochemistry, The John Curtin School of Medical Research, from February, 1969 to October, 1972, during the tenure of an Australian National University Scholarship.

The general abbreviations used without definition in this thesis, are those recommended by Biochimica et Biophysica Acta, Information for Contributors, Revised Edition (1971).

All experimental work reported in this thesis was performed by the author. Work which was carried out in conjunction with other colleagues is acknowledged in the text.

Jeannette D. Lawrence.

Candidate's Signature.

ACKNOWLEDGEMENTS.

I would like to thank my supervisors Professor F.W.E. Gibson and Dr. J.F. Morrison for helpful discussion and guidance during the course of this work.

My sincere thanks go to Dr. A.B. Roy who so very willingly carried out the ultracentrifugation analyses and to Dr. D.C. Shaw who prepared the peptide maps. To Mr. D. Abigail, I would like to express my sincere thanks for his readiness and co-operation in the growth of cells and preparation of cell extracts, and I would like to thank Mr. L.B. James for carrying out the amino acid analysis.

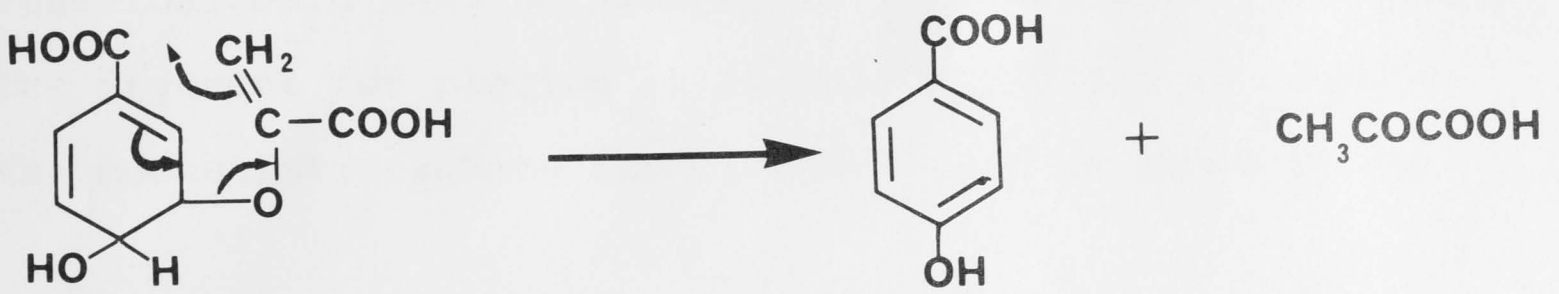
Finally, I would like to thank my sister for typing this thesis and my husband for being most tolerant, especially during the closing stages of this thesis.

ERRATA

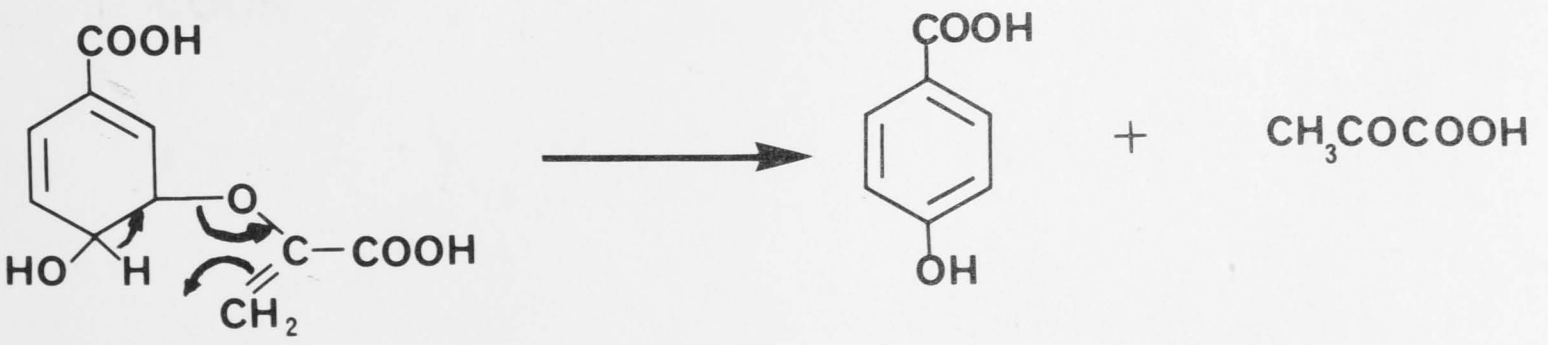
1.	Page (viii) line 20.	For "prepartion" read "preparation"
2.	Page 10 line 11.	For "tp" read "to"
3.	Page 23 lines 12,13, and 14.	The medium described as "Medium 56" is incorrect and should be described as "a modification of Medium 56"
4.	Page 29 line 19.	Insert the word "in" after "4°C"
5.	Page 29 line 21.	For "approximation" read "approximately"
6.	Page 34 line 12.	For "chorisimate" read "chorismate"
7.	Page 43 lines 11,12.	"The minimal medium used is described in MATERIALS AND METHODS, Section 2.1."
8.	Page 53 line 2.	For "Ubiquinone" read "2-octaprenyl-phenol"
9.	Page 67 line 2.	For "Figure 2.3" read "Figure 3.3"
10.	Page 72 line 25.	For "Sephadex G-150" read "Sephadex G-100"
11.	Page 86 line 1.	For "approximate" read "apparent"
12.	Page 88 line 19.	For "chlordie" read "chloride"
13.	Page 100 Table VII.	At 10 min the concentration of 4-hydroxybenzoate should be 0.01 µmoles/ml
14.	Page 114 line 13.	For "machanism" read "mechanism"
15.	Figure 3.12 line 9.	For "approximate" read "apparent"
16.	Throughout	For "supernatant" read "supernatant solution"
17.	Throughout	For "aliquot" read "sample"

FOREWORD.

The enzyme converting chorismate (I) to 4-hydroxybenzoate (II) and pyruvate (III) has not been previously named. The two mechanistic schemes shown below indicate that in each case the C-O bond is broken thus releasing a pyruvate moiety.



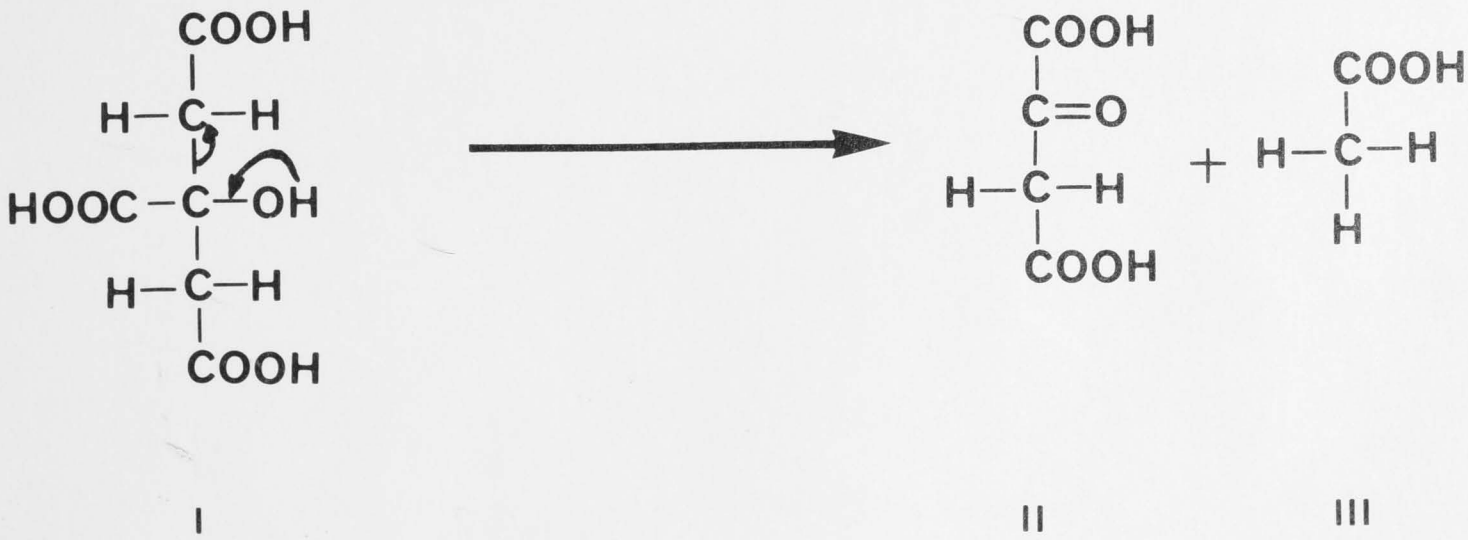
I II III



An enzyme which is capable of splitting a C-O bond is called a lyase. This is in agreement with the Report of the Commission on Enzymes of the International Union of Biochemistry (1961) which states that:-

> 'Enzymes removing groups from substrates non-hydrolytically, leaving double bonds (or adding groups to double bonds) will be called "lyases" in the systematic nomenclature.'

The sub-group into which the enzyme carrying out this reaction could best be placed, is the keto-acid lyase group. The argument for placing it in this sub-group is based on the mechanistic scheme for citrate lyase as shown below.



I II III

It is seen that the hydroxyl group of citrate (I) is deprotonated and this unstable situation yields the more stable ketone thus breaking the C-C bond to form oxaloacetate (II) and acetate (III). From this reaction mechanism, the systematic name given to the enzyme is citrate oxaloacetate lyase, since a ketone group is formed as a result of the hydroxyl group being deprotonated.

Similarly, this enzyme produces a keto-acid, pyruvate. On this reasoning, therefore, the enzyme has been given the systematic name of chorismate pyruvate lyase. The trivial name suggested for this enzyme is 'chorismate lyase', which name will be used in this thesis.

ABBREVIATIONS.

Throughout this thesis, each Figure and Table is presented on a separate page and follows the page of the text on which the first reference to it has been made.

The following abbreviations have been used:-

Dansyl	5-Dimethylamino-naphthalene sulphonyl
HEPES	N-2Hydroxyethylpiperazine-N' -2-ethanesulphonic acid.
TES	N-Tris(hydroxymethyl)methyl-2-amino ethane sulphonic acid.
TEA	Triethanolamine

SUMMARY.

Chorismate lyase was purified to homogeneity from extracts of Escherichia coli, AN58. The purification of the enzyme was carried out as it was of interest to determine if this vitamin enzyme involved in the biosynthesis of ubiquinone, a well-known respiratory compound, was present in the cell in normal amounts and/or possessed a high or low turnover number.

The genetic and biochemical aspects of a mutant strain of E. coli which lacked the ability to form 4-hydroxybenzoate was investigated and it was found that the gene which coded for chorismate lyase was probably the structural gene. Regulation of chorismate lyase activity in whole cells indicated that ubiquinone did not affect the level of activity whereas other indications suggested that 2-octaprenyl-phenol or an earlier biosynthetic intermediate could repress the level of chorismate lyase.

The protein was shown to be homogeneous by several criteria. Sedimentation velocity experiments were carried out and results showed the presence of only one symmetrical peak. Polyacrylamide gel electrophoresis of the enzyme in the presence of 0.1% sodium dodecyl sulphate yielded only a single polypeptide chain. The molecular weight was determined by two methods, namely by equilibrium

sedimentation experiments and by gel filtration and the results from gel filtration experiments indicated a molecular weight of 15,800. Fingerprints of tryptic peptides obtained from digests of chorismate lyase indicated that the enzyme contained 14-18 peptides which is a little higher than the range predicted from the amino acid composition, for a protein with a molecular weight of 15,800. Reasons are given why this aspect of the work requires further investigation.

General kinetic properties of chorismate were studied and the results indicated that the Km for the reaction was high while the Ki for 4-hydroxybenzoate was very low. Results showed that low concentrations of 4-hydroxybenzoate could competitively inhibit the reaction. Other compounds which were structurally similar to either the substrate or product of the reaction were tested for their affect on the reaction. Allosteric modifiers and various chemical reagents were also tested for their effect.

Difficulty has been incurred in the prepartion of this enzyme due to (i) the low levels of activity in cell-free extracts (ii) the effects observed on concentrating protein solutions and (iii) the effects of using high and low ionic strength buffer. The complex behaviour of chorismate lyase was eventually understood and further

investigations of its properties were carried out. The enzyme was eventually shown to possess an extremely low turnover number which is to be expected because it is a vitamin enzyme.

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SECTION 1. GENERAL INTRODUCTION.

The shikimate pathway for the biosynthesis of aromatic compounds was elucidated by several groups of workers, as exemplified by Davis (1955), Sprinson (1960), and Gibson and Gibson (1964). The general outlines of the pathways to the aromatic amino acids, phenylalanine, tyrosine, and tryptophan have been known for some time and have been reviewed by Umbarger and Davis (1962) and Gibson and Pittard (1968). The original discovery of 4-hydroxybenzoate as a bacterial vitamin was first shown by Davis (1950).

Mutants of Escherichia coli were isolated by Davis (1950) and found to respond to the four supplements, tyrosine, phenylalanine, tryptophan and 4-aminobenzoate. Such mutants were called multiple aromatic auxotrophs since the metabolic block occurred in the common pathway. The quantitative requirements for the three amino acids were similar to those mutants with single requirements for these compounds. However, growth was very slow with low concentrations of 4-aminobenzoate (0.005 µg per ml), although this concentration was sufficient for optimal growth of a mutant blocked specifically in 4-aminobenzoate synthesis. Higher concentrations of 4-aminobenzoate (up to 1 µg per ml) provided more rapid, yet less than normal growth of the mutant. It was shown that normal growth-rate was obtained when shikimate, a known precursor of the above four metabolites, was included in the medium. It was therefore postulated by Davis (1950) that another compound, which could readily be formed from shikimate,

was responsible for recovery of normal growth. The compound was subsequently shown to be 4-hydroxybenzoate and a concentration as low as 0.01 µg per ml supported rapid growth in the presence of the other four supplements. The high order of activity shown by 4-hydroxybenzoate led Davis to conclude that this compound should be considered a bacterial vitamin. 4-Hydroxybenzoate, like 4-aminobenzoate, was found in significant concentrations in yeast autolysate, in filtrates from cultures of wild type bacteria but not in liver extract. (It was also shown by Davis (1950) that 4-hydroxybenzoate is present in Whatman No. 1 chromatography paper.)

Davis (1952) subsequently showed that the requirement for 4-hydroxybenzoate as a growth factor was not an absolute one. Mutants of E. coli, Aerobacter aerogenes, Salmonella typhimurium, and Bacillus subtilis, that required two, three, four or five aromatic compounds, were shown to be blocked in the synthesis of those precursors that are common to all five of the compounds. The more complete the block, the greater the number of growth requirements needed; an increasing inability to synthesize the common precursors leads to successive growth requirements in the following order: tyrosine, phenylalanine, tryptophan, 4-aminobenzoate and finally, 4-hydroxybenzoate.

Briefly discussing the various biosynthetic pathways, the common pathway involves the condensation of two products of carbohydrate metabolism, phosphoenolpyruvate and erythrose-4-phosphate, to give a straight chain seven carbon compound

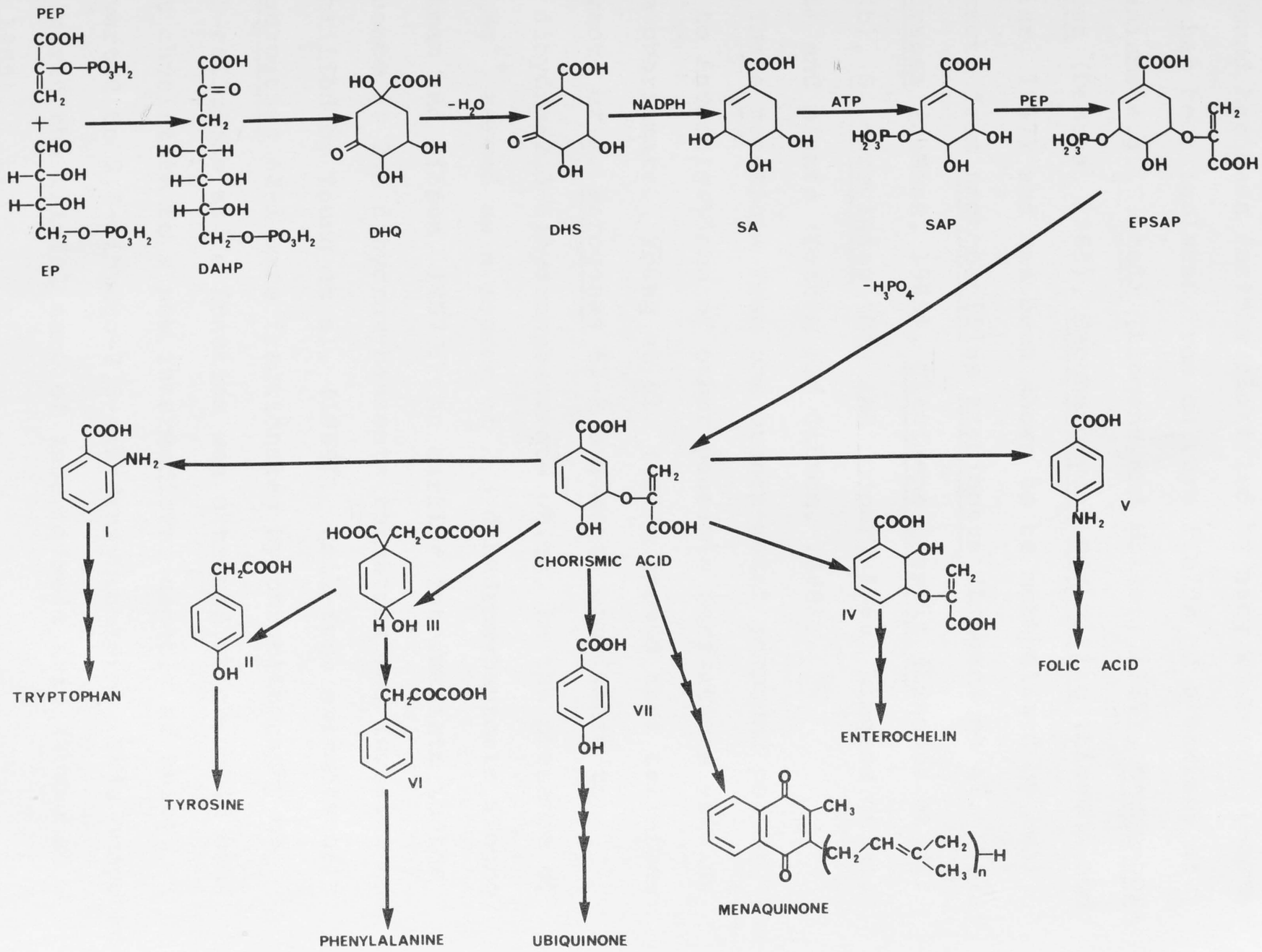
(Figure 1.1). This compound is then cyclized and undergoes a series of reactions through shikimate to chorismate. Before 1964, little was known about the branch-point compound. It was known, however, that 3-enolpyruvylshikimate-5-phosphate was an intermediate in the common pathway (Levin and Sprinson, 1960, 1964) and that this compound gave rise to three other compounds involved with aromatic biosynthesis, namely anthranilate, phenylpyruvate, and 4-hydroxyphenylpyruvate (Gibson et al. 1962; Rivera and Srinivasan, 1962). With the isolation and identification of chorismate (Gibson and Gibson, 1962, 1964; Gibson, 1964), the branch-point compound from which all other aromatic pathways diverged was established.

The isolation of chorismate was achieved by using a triple mutant of A. aerogenes 62-1 in which tryptophan, and tyrosine were essential growth factors, and phenylalanine stimulated growth. Cell-free extracts of this strain were shown to form chorismate from a mixture of shikimate, ribose-5-phosphate, NADH, ATP and Mg^{2+}. Cell-free extracts of a multiple aromatic auxotroph A. aerogenes 170-44 with a metabolic block immediately after 3-enolpyruvylshikimate-5-phosphate, could not only enzymically convert chorismate to anthranilate, (in the presence of glutamine) but also convert chorismate to prephenate (and so to phenylpyruvate and 4-hydroxyphenylpyruvate), 4-aminobenzoate and 4-hydroxybenzoate (Gibson and Gibson 1962, 1964; Gibson, 1964; Gibson et al. 1964).

FIGURE 1.1 General outlines of pathways for the formation of aromatic amino acids and vitamins in E. coli.

Abbreviations: PEP, phosphoenolpyruvate; EP, erythrose-4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonic acid 7-phosphate; DHQ, 5-dehydroquinic acid; DHS, 5-dehydroshikimic acid; SA, shikimic acid; SAP, shikimic acid 5-phosphate; EPSAP, 3-enolpyruvylshikimic acid 5-phosphate; I. anthranilic acid; II. 4-hydroxyphenylpyruvic acid; III. prephenic acid; IV. isochorismic acid; V. 4-aminobenzoic acid; VI. phenylpyruvic acid; VII. 4-hydroxybenzoic acid.

FIGURE 1.1


PEP
EP
DAHP
DHQ
DHS
SA
SAP
EPSAP
NADPH
ATP
PEP
$-H_2O$
$-H_3PO_4$
CHORISMIC ACID
I
TRYPTOPHAN
II
TYROSINE
III
PHENYLALANINE
VI
VII
UBIQUINONE
IV
ENTEROCHELIN
V
FOLIC ACID
MENAQUINONE

Since this time the region around the branch-point compound has been further clarified by many workers. Chorismate has been isolated from culture fluids of a variety of organisms e.g. E. coli (Lingens and Müller, 1967), Neurospora crassa (DeMoss, 1965), Saccharomyces cerevisiae (Lingens and Müller, 1967) and has been shown to be metabolized by cell extracts from Lactobacillus arabinosus (Lingens et al. 1967a), N. crassa (DeMoss, 1965), Claviceps paspali (Lingens et al. 1967b), S. cerevisiae (Doy and Cooper, 1966; Lingens et al., 1966) and plants (Cotton and Gibson, 1968).

These reactions from the branch-point compound pointed the way to investigations of other possible derivatives arising from chorismate. Young et al. (1967a)showed that cell-free extracts of A. aerogenes 62-1 converted chorismate to 2,3 dihydro-2,3-dihydroxybenzoate which, in the presence of NAD, Mg^{2+}, served as a source of 2,3 dihydroxybenzoate (Young, Jackman and Gibson, 1967 b). An earlier intermediate in the sequence of 2,3 dihydroxybenzoate formation was isolated and identified by Young et al. (1969). Cell-free extracts of A. aerogenes 62-1 were fractionated by chromatography on DEAE-cellulose and a fraction was obtained which could convert chorismate to a new intermediate, capable of being converted to 2,3-dihydro-2,3-dihydroxybenzoate. This compound was given the trivial name of isochorismic acid (Young et al. 1969).

Subsequently, Brot and Goodwin (1968) isolated an enzyme from extracts of a methionine-vitamin B_{12} auxotroph of E. coli K-12 which catalyzed the synthesis of 2,3-dihydroxy-benzoylserine from 2,3-dihydroxybenzoate and L-serine in the presence of ATP. The enzymic activity only appeared during the latter stages of growth and could not be detected when the organism was grown in a glucose, methionine salts medium containing $FeSO_4$. The appearance of enzymatic activity from cells that were grown in the presence of iron, was dependent upon the presence of a chelating agent and the de novo synthesis of protein. Further, 2,3-dihydroxy-N-benzoylserine has been isolated and identified in E. coli by O'Brien et al. (1969). The end product of the pathway in E. coli involving 2,3-dihydroxybenzoate has been identified by O'Brien and Gibson (1970) as a cyclic derivative of 2,3 dihydroxybenzoate, and has been named enterochelin. A similar compound has been isolated in Salmonella typhimurium by Pollack and Neilands (1970) and called enterobactin. Both enterobactin and enterochelin are iron sequestering agents.

Apart from chorismate being metabolized along the various pathways (Figure 1.1), it is unstable under physiological conditions. Gibson (1964), and Gibson and Gibson (1964) showed that chorismate under these conditions decomposes to a mixture of compounds, namely 4-hydroxybenzoate and prephenate, the latter compound giving phenylpyruvate in acid solution.

Before 1964, little was known of the biosynthetic origin of ubiquinone and menaquinone. The initial observation of Rudney and Parson (1963) that ^{14}C-labelled 4-hydroxybenzaldehyde was incorporated into the benzoquinone ring of ubiquinone in the photosynthetic bacterium, Rhodospirillum rubrum, established the first possible intermediate in the biosynthesis of ubiquinone. Later, Parson and Rudney (1964) showed that ^{14}C-4-hydroxybenzoate, as well as the aldehyde were able to be incorporated into ubiquinone in Azotobacter vinelandii, Baker's yeast and rat kidney. These two compounds were also shown to be incorporated into R. rubrum (Parson and Rudney 1965).

Since this time, the number of organisms which are known to incorporate 4-hydroxybenzoate into ubiquinone has increased. Cox and Gibson (1964) found that 4-hydroxybenzoate and shikimate were incorporated into ubiquinone in E. coli. Multiple aromatic auxotrophs of E. coli, when grown on a glucose mineral salts medium supplemented with the aromatic amino acids and 4-aminobenzoate, only formed ubiquinone when 4-hydroxybenzoate was present (Cox and Gibson 1966). However, in similar experiments with A. aerogenes, ubiquinone was formed in the absence of 4-hydroxybenzoate, suggesting that an alternative pathway to ubiquinone could exist in these cells. The results of experiments with cell-free extracts of A. aerogenes indicated that 4-hydroxybenzoate was formed from tyrosine (Cox and Gibson, 1966), (Figure 1.2). Although 4-hydroxyphenylpyruvate may be spontaneously converted to

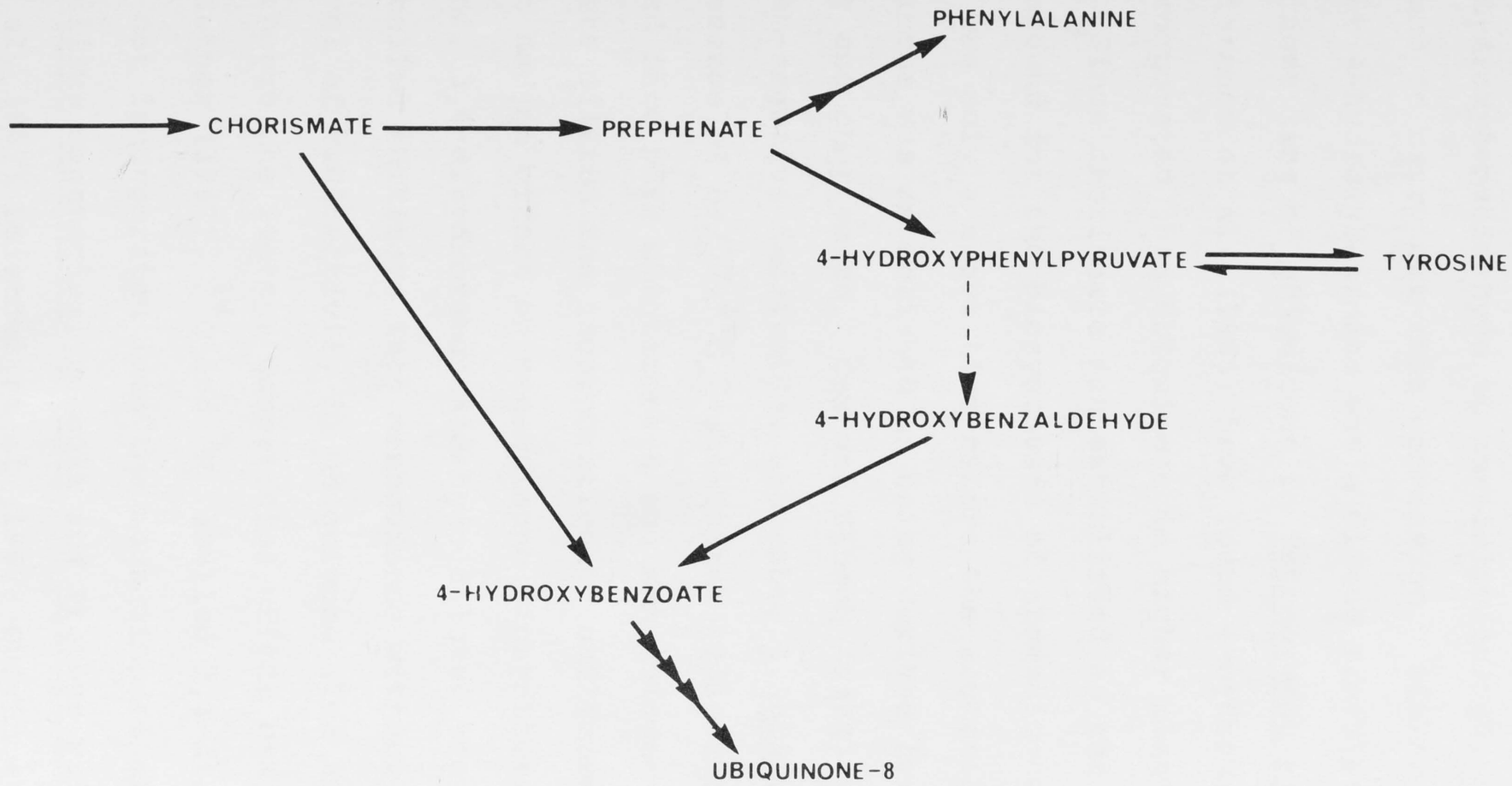


FIGURE 1.2 Outline of probable pathways of the biosynthesis of 4-hydroxybenzoate in E. coli and A. aerogenes. The broken line represents a pathway that has not been confirmed by experiments with cell-free extracts. E. coli could not convert 4-hydroxybenzaldehyde into 4-hydroxybenzoate. --- Taken from Cox and Gibson (1966).

4-hydroxybenzaldehyde at physiological pH, no enzyme has been found to carry out this conversion. Miller (1965) showed that 4-hydroxybenzoate was a direct precursor of the benzoquinone ring of ubiquinone in Tetrahymena pyriformis while Whistance et al. (1966) have found 4-hydroxybenzoate was incorporated into ubiquinone in higher plant tissue.

Since chorismate was established as the branch-point compound for the biosynthesis of aromatic compounds in bacteria, it was only a short time before the aromatic nucleus of ubiquinone was established as being derived from the common pathway and chorismate. Cox and Gibson (1964) showed that when the gram-negative facultative anaerobe, E. coli, was grown in the presence of D-[G-^{14}C] shikimate, radioactivity was incorporated into both ubiquinone-8 and menaquinone-8. 4-Hydroxybenzoate diluted the incorporation of shikimate into ubiquinone but had no effect on menaquinone biosynthesis. On the other hand, 3,4-dihydroxybenzaldehyde diluted the incorporation of labelled shikimate into menaquinone without affecting the level of radioactivity in ubiquinone (Cox and Gibson, 1966). Although the isotope competition effect has been confirmed by Leistner (1967), ^{14}C and ^{3}H labelled 3,4-dihydroxybenzaldehyde is not incorporated into the menaquinones of A. aerogenes, Bacillus megaterium, E. coli and Proteus vulgaris (Campbell et al. 1967; Leistner et al. 1967; Guérin et al. 1970).

On studying the biosynthesis of menaquinone, Cox and Gibson (1966) found that multiple aromatic auxotrophs of

A. aerogenes blocked between 3-enolpyruvylshikimate-5-phosphate and chorismate were unable to synthesize menaquinone or ubiquinone. A possible precursor in the biosynthesis of menaquinone was suggested by Leistner et al. (1967) who showed that ^{14}C-α-naphthol was incorporated into menaquinone. Since then experiments have been reported which show that radio-activity from α [1 - ^{14}C] naphthol is incorporated into menaquinones by the Gram-positive bacterium Staphylococcus aureus and the Gram-negative bacterium A. aerogenes (Guérin et al. 1970; Hammond and White, 1969), but not the Gram-positive bacteria B. megaterium, B. subtilis, Micrococcus lysodeikticus, Mycobacterium phlei and the Gram-negative bacteria E. coli, Proteus mirabilis and P. vulgaris (Brown et al., 1968; Ellis and Glover, 1968; Guérin et al. 1970). These anomalous results were explained by Threlfall (1971) who stated that α-naphthol was an abnormal precursor and that those organisms which could utilize it possessed the necessary enzyme to convert it to 1,4 naphthoquinol.

More recently Dansette and Azerad (1970) have proposed a scheme for the biosynthesis of menaquinone. This involves the condensation, coupled with the dehydration of chorismate, of a C_4 derivative of glutamate followed by a simple elimination reaction to form O-succinyl benzoate. A more favoured hypothesis is the attack of the same C_4 derivative on isochorismate. To support their proposals, Dansette and Azerad (1970) showed that when multiple aromatic auxotrophs of E. coli, blocked in the early steps of aromatic biosyn-

thesis, were grown on an aromatic supplement and O-succinyl-benzoate, the same amount of menaquinone-8 was formed as when the same auxotrophs were grown in the presence of shikimate. When a mutant strain of E. coli was blocked after shikimate, only O-succinylbenzoate was effective. Finally, these workers synthesized O-succinyl /carboxy-^{14}C/ benzoic acid and showed that the radio-activity was incorporated into menaquinone-9 by M. phlei, demethylmenaquinone-8 and menaquinone-8 by A. aerogenes and E. coli, and a number of other plants. Dansette and Azerad (1970) proposed that O-succinyl-benzoate was cyclized to form 1,4-dihydroxy-2-naphthoate, the first napthalenic compound on the pathway and this was then decarboxylated to give 1,5 naphthoquinol. Other than these lines of evidence nothing more is known about the biosynthesis of menaquinone.

A pathway for the complete biosynthesis of ubiquinone has been proposed in E. coli by Friis et al. (1966) and is shown in Figure 1.3. However, at the time that the present work was commenced, only a few of the compounds proposed in this scheme had been isolated and fully characterized. There have been two main approaches used to isolate and identify intermediates in the pathways leading to the biosynthesis of ubiquinone. One approach that has been taken by several groups of workers is that in normal cells, small quantities of biosynthetic intermediates may be present (Olsen et al., 1966; Imamoto and Senoh, 1967; Whistance, Brown and Threlfall, 1969;

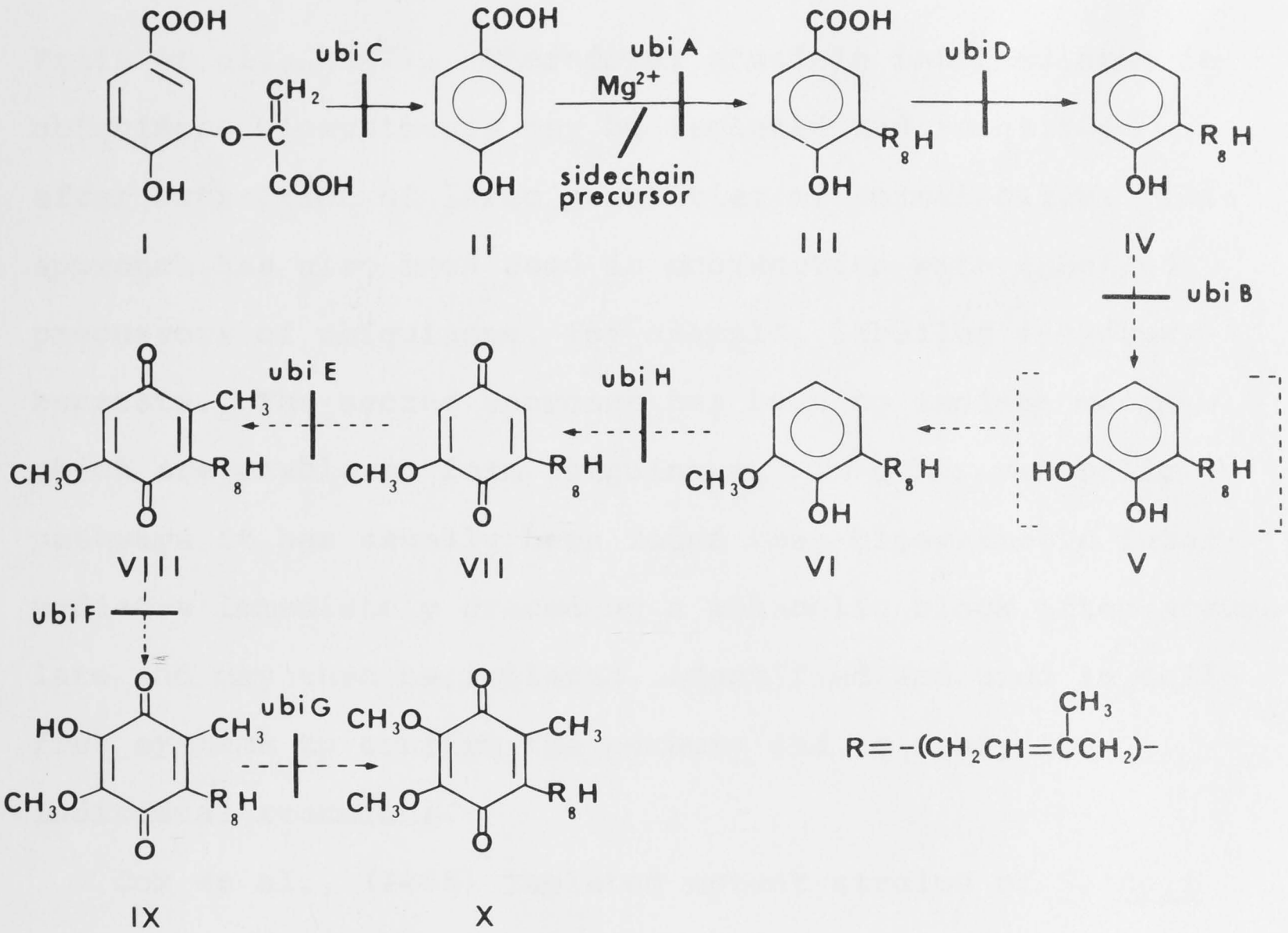


FIGURE 1.3 The possible pathway of ubiquinone biosynthesis in E. coli showing the positions of the ubi genes and the reactions which are affected by these mutations. The broken lines represent reactions that have not been confirmed by experiments with cell-free extracts. The compounds are :

I. chorismate; II. 4-hydroxybenzoate;

III. 4-hydroxy-3-octaprenylbenzoate;

IV. 2-octaprenylphenol; V. 6-hydroxy-2-octaprenyl-phenol; VI. 6-methoxy-2-octaprenylphenol;

VII. 6-methoxy-2-octaprenyl-1,4-benzoquinone;

VIII. 6-methoxy-3-methyl-2-octaprenyl-1,4-benzo-quinone; IX. 5-hydroxy-6-methoxy-3-methyl-2-octa-prenyl-1,4-benzoquinone; X. ubiquinone.

Friis et al., 1967). Therefore, possible intermediates in ubiquinone biosynthesis may be isolated and identified after extraction of large quantities of normal cells. This approach has also been used in conjunction with labelled precursors of ubiquinone, for example, labelled 4-hydroxy-benzoate. The second approach has been to isolate mutants which are unable to form ubiquinone. In other metabolic pathways it has usually been found that biosynthetic intermediates immediately preceding a metabolic block often accumulate and may then be isolated, identified and used in cell-free systems tp confirm the pathway and to study the individual reactions.

Cox et al., (1968) isolated mutant strains of E. coli K-12 unable to form ubiquinone , using a technique involving an indirect selection procedure. It was assumed that ubiquinone was an essential component in the electron-transport chain between certain oxidizable substrates and molecular oxygen. The utilization of compounds, such as malate or succinate, as sole source of carbon must rely on hydrogen transfer to oxygen for the cell to obtain energy and intermediates for biosynthesis (a discussion of the role of ubiquinone in electron transport is given later). On the other hand a strain lacking ubiquinone should grow with glucose as sole source of carbon because E.coli is a facultative anaerobe and can grow by fermentative mechanisms. Therefore, ubiquinone-deficient strains of E. coli were selected for their ability to grow fermentatively on glucose, and for

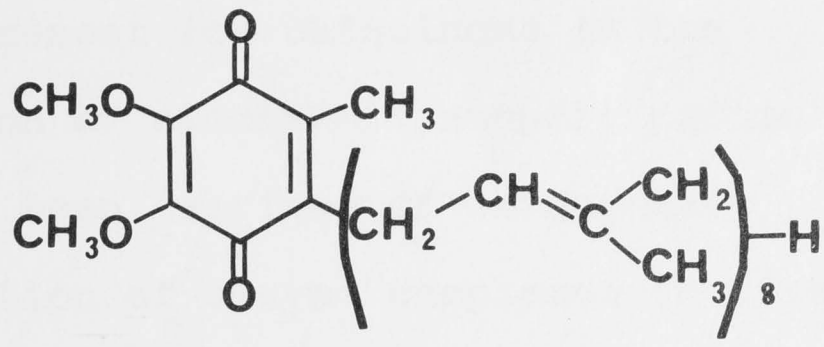
their inability to grow on a reduced substrate such as malate or succinate as sole source of carbon. A number of ubiquinone mutants have now been isolated by the above procedure and genetically and biochemically characterized (Cox, Gibson and Pittard, 1968; Cox, Young et al., 1969; Young et al., 1971; Stroobant et al., 1972; Young, et al., 1972). The reactions which are affected by the ubiA, ubiB, ubiD, ubiE and ubiF, ubiG and ubiH mutations are shown in the proposed scheme. At the commencement of this study only one of the reactions had been studied in cell-free extracts of A. aerogenes and this was the conversion of chorismate to 4-hydroxybenzoate (Gibson and Gibson, 1962, 1964).

At this point, it is convenient to discuss the quinones, ubiquinone and menaquinone, as the function of these compounds is important and leads to an understanding of what happens when these compounds are absen in ubiquinone-deficient mutants. Ubiquinone, previously called coenzyme Q, was first isolated from one of a number of lipid fractions of horse intestine mucosa, (Festenstein et al. 1955). One of these lipid fractions yielded an absorption maxima at 272 nm in cyclohexane. Morton (1956) and Crane et al. (1957) also isolated a compound having similar properties to the lipid fraction of Festenstein and coworkers. Crane et al. (1957) recognized the quinonoid properties of this compound and therefore its possible involvement as an oxido-reduction compound in the respiration process. The correct

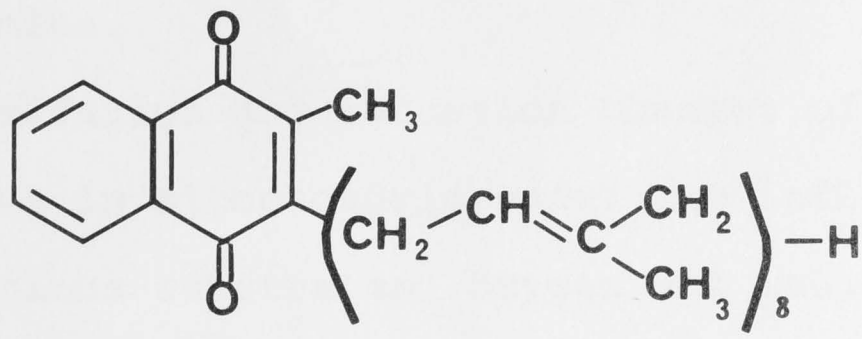
complete structure was not established until 1958 by several groups of workers, namely Fahmy et al. (1958), Lester et al. (1958) and Wolf et al. (1958).

Ubiquinone is a 2,3 dimethoxy-5-methyl-1,4 benzoquinone with an isoprenoid side chain of varying length. The number of isoprenyl units is indicated e.g. ubiquinone-8 in E. coli (Figure 1.4). One type of ubiquinone usually predominates in a particular species, although other homologues may be present. Ubiquinone has been shown to occur in plants, animals and microorganisms (Lester and Crane, 1959). The most common, naturally occurring ubiquinone in higher animals and plants, is ubiquinone-10 which has ten isoprenoid units. Other members of the group have side chains varying from six to nine isoprenoid units (Crane and Sun, 1972). Ubiquinone is found in varying quantities in cells from different organisms. Lester and Crane (1959) collected data on the concentration of ubiquinone from a large number of animals, plants and microorganisms and generally concluded that the amount of ubiquinone present could be correlated with the respiratory capacity of the tissue or organism in question, e.g. cardiac tissue was always found to contain more ubiquinone than skeletal muscle which is in keeping with the higher respiratory rates of the cardiac tissue.

The function of the quinones in electron transport has been reviewed in several articles (Chance, 1965; Redfearn, 1966; Crane and Low, 1966; Kröger and Klingenberg, 1967; Crane, 1968; and Crane and Sun, 1972). The approaches



UBIQUINONE - 8



MENAQUINONE - 8

FIGURE 1.4 Structure of UBIQUINONE-8 and MENAQUINONE-8 found in E. coli.

taken to investigate and provide evidence for the role of ubiquinone in electron transport include:-

(a) the requirement for ubiquinone in the respiration of electron transport particles that have been deprived of ubiquinone.
(b) the isolation of enzyme complexes involved with the respiration process and particularly those which catalyse the oxidation and reduction of ubiquinone.
(c) the oxidation and reduction by mitochondrial particles of ubiquinone that has been extracted and isolated from the same particles.
(d) the oxidation and reduction changes of ubiquinone in mitochondria under the influence of various substrates, oxygen and inhibitors.

Kröger and Klingenberg (1967) have made extensive studies of the redox potentials of the respiratory carriers in the aerobic state and concluded that ubiquinone functions between the flavoproteins and the cytochromes. A diagram of the currently accepted sequence of electron transport carriers in mitochondria is shown in Figure 1.5, together with the sites of some of the better known respiratory inhibitors.

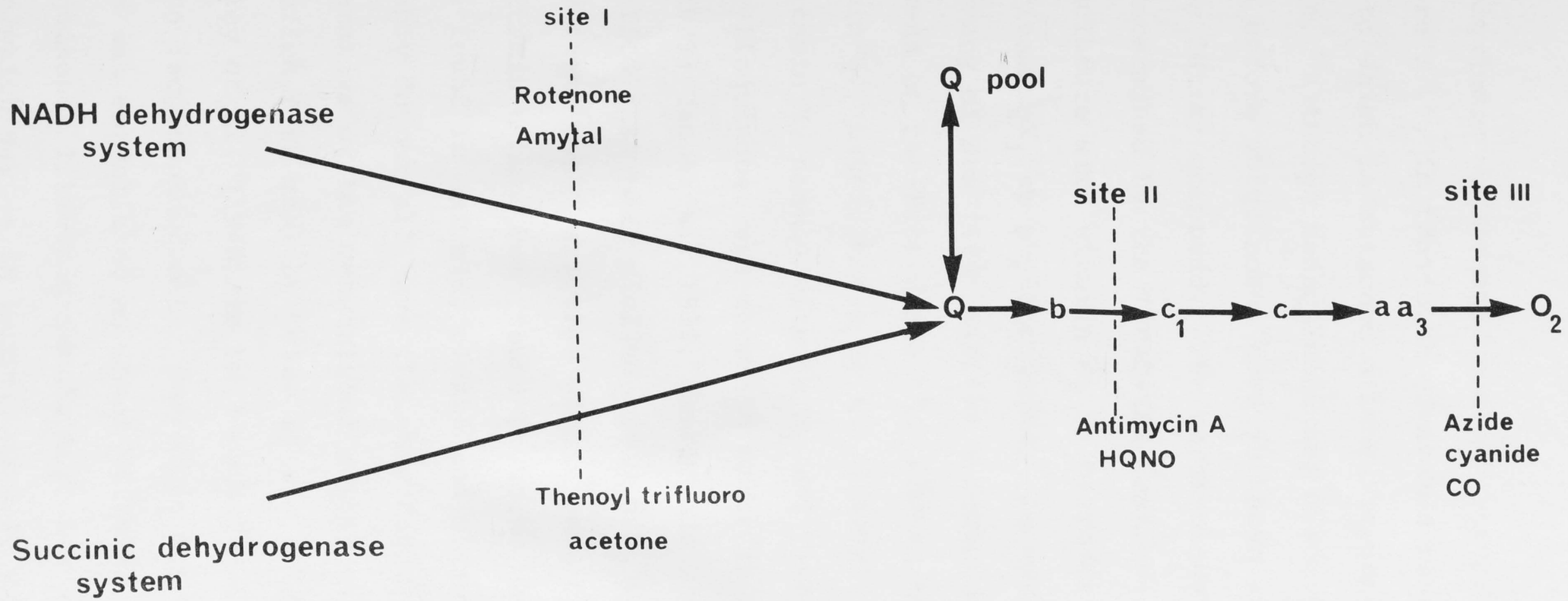


FIGURE 1.5 Schematic representation of the sequence of electron transfer in mitochondria, showing the three sites of phosphorylation and the sites of action of some respiratory inhibitors.
Taken from Klingenberg (1968) and Crane (1968).

Ubiquinone and menaquinone are structurally related (Figure 1.4), in that both compounds possess a quinone ring to which is attached a long isoprenoid chain of varying length. Although menaquinone was discovered some twenty years before ubiquinone, more is known about the biosynthesis of the latter compound. The terminology for the vitamins K was determined by the IUPAC-IUB Commission on Biochemical Nomenclature with vitamin K_2 to be referred to as menaquinone and vitamin K_1 as phylloquinone. It was the original discovery of Dam (1935 a,b) of a coagulation factor in chickens on fat-free diets that led to the discovery of vitamin K. Later, McKee et al. (1939a) discovered two types of vitamin K, namely vitamin K_1 and vitamin K_2. Vitamin K_1, or phylloquinone, was shown to occur in alfalfa (Binkley et al. 1939; Dam et al. 1939; Karrer and Geiger, 1939), while putrefied fish meal yielded vitamin K_2 or menaquinone. Since then, many varieties within each of these two types of vitamin K have been found to occur naturally. The vitamins K are found in animals, plants and microorganisms but they probably do not all have the same function. The first representative of the menaquinone series was isolated from putrefied fish meal by McKee et al. (1939b), and shown by Binkley et al. (1940) to be 2-methyl-1,4-naphthoquinone with a long isoprenoid chain. The exact number of isoprenoid units was established as seven by Isler et al. (1958). Menaquinone, like ubiquinone, also has an isoprenoid side-chain which varies in length, usually with one form of the series predominating in any one species.

The antihaemorrhagic properties of vitamin K_2 in animals have long been known to be associated with the synthesis of prothrombin in the liver. However, in those organisms that are able to produce vitamin K_2 and yet do not possess a liver, it is therefore concomitant to say that vitamin K_2 cannot be involved in prothrombin formation. Therefore it must have some other function. Martius (1966) has suggested that in the cell metabolism of all organisms, vitamin K_2 must have a specific function similar to other vitamins, since lower animals also require the vitamin (Martius et al. 1965). These authors have shown that several invertebrates, such as earthworm, snail and some insects, are able to convert menadione into vitamin K_2. These facts suggest that there is a basic role for menaquinone in cell metabolism.

Quinones in bacteria have been shown to be localized in the sub-cellular structures, namely in the cytoplasmic membrane (Mitchell and Moyle, 1956; Weibull and Bergström, 1958) and these subcellular structures are known to be involved in biological processes of oxidation and reduction (Brodie, 1957; Brodie et al., 1958; Bishop et al., 1962; Baum and Dolin, 1963; White, 1965; Kröger and Dadak , 1969). The fact that quinones are found on fractionation of the cells in the membrane fraction, together with membrane-bound cytochromes, suggested that the quinones may serve as electron carriers in electron transport (Bishop and King, 1962; Smith, 1968; Kröger and Dadak, 1969).

Morton (1961) has also produced data on the distribution of ubiquinone and menaquinone in microorganisms and showed that both quinones are implicated in aerobic respiration. In agreement with this data, Rudney and Sugimura (1961) showed that when yeast cells were grown under anaerobic conditions, ubiquinone-6 was found to occur in smaller amounts than when grown aerobically. On aerating anaerobically grown yeast in a glucose phosphate medium, the ubiquinone content increased five to ten fold. Bishop et al. (1962) have shown that most of the aerobic bacteria contain either ubiquinone or menaquinone, or both. Gram-positive organisms contain menaquinone only, while gram-negative organisms are of two types, those containing ubiquinone and those containing both ubiquinone and menaquinone.

Table 1 shows some of the respiratory components of the membranes in various types of bacteria and these are compared with those of the mitochondrial membrane. On a protein basis, the level of the quinones and cytochrome b, as well as the NADH-oxidase activities in the bacterial strains, are of the same order of magnitude as those of the mitochondria from beef heart (Table 1). This indicates the functional similarity with respect to the respiration of the bacterial cytoplasmic membrane and the mitochondrial membranes.

Although E. coli has both ubiquinone and menaquinone, Bishop et al. (1962) demonstrated that the menaquinone content did not vary when E. coli was grown either aerobically or

TABLE 1.

CONTENTS OF RESPIRATORY COMPONENTS IN MEMBRANES*

Source	Ubiquinone (μmoles/g protein)	Menaquinone (μmoles/g protein)	Cytochrome b (μmoles/g protein)	NADH-oxidase activity (μatoms O_2 per min/g protein)
Mitochondria beef heart	6	0	0.9	850
Micrococcus denitrificans (NCIB 8944)	1.5	0	0.18	570
B.megaterium (ATCC 14581)	0	5.4	0.87	985
Proteus rettgeri	5.4	3.4	0.46	657
E. coli	4.7	0.67	0.19	560

* Taken from Kröger and Klingenberg (1970) and Cox et al. (1970).

anaerobically. This is in contrast to the work of Polglase et al., (1966) who showed that menaquinone is present only in small amounts in E. coli when the cells are grown aerobically and rises to higher levels during anaerobic growth. This is in agreement with the earlier work of Lester and Crane (1959) who showed that when E. coli B_4 was grown aerobically, ubiquinone and menaquinone were present, but when grown anaerobically, only menaquinone was present.

Bishop and King (1962) demonstrated that when cells of E. coli were mechanically broken, cell residues were obtained which possessed both ubiquinone and menaquinone, whereas Kashket and Brodie (1963a,b,) were able to separate ubiquinone and menaquinone by fractionating the cell-free preparation of E. coli. The 'heavy' fraction contained ubiquinone and most of the succinoxidase activity while the 'lighter ' fraction contained both ubiquinone and menaquinone in the ratio of 10:1. This light fraction was found to oxidise NAD-linked substrates with coupled phosphorylation. Kashket and Brodie (1963b) therefore proposed that in E. coli ubiquinone was linked with succinoxidase activity while menaquinone was linked with NADH-cytochrome b reductase.

More recently in experiments to determine the function of ubiquinone in E. coli, Cox et al. (1970) have reported that ubiquinone-deficient strains when grown under aerobic conditions have greatly impaired NADH and lactate oxidase activities. These activities were restored by the addition of ubiquinone (Q-1). On comparison of the percentage

reduction of flavin, cytochrome b, and cytochrome a_2 in the aerobic steady state in membranes from normal and ubiquinone-deficient strains, and the effect of respiratory inhibitors on these percentages, ubiquinone was shown to function at more than one site in the electron-transport chain. A scheme was therefore proposed in which ubisemiquinone, complexed to an electron carrier, functioned in at least two positions in the electron-transport sequence before and after cytochrome b_1. For efficient aerobic electron transport, ubiquinone was required in considerable molar excess over other components of the electron transport system (Newton et al., 1972). A comparison of aerobic steady states of cytochrome b_1 in membranes from normal, partially ubiquinone-deficient, and completely ubiquinone-deficient strains, indicated that the proposed sites of ubiquinone function in the scheme mentioned previously (Cox et al., 1970) are equally affected by the partial depletion of the quinone (Newton et al., 1972). Further, these workers suggested that ubiquinone could be involved as a mobile substrate in additional step(s) between the electron transport chain and adenosine triphosphate formation.

A mutant strain of E. coli deficient in menaquinone, or vitamin K_2 has been isolated by Newton et al., (1971). Under aerobic conditions this mutant strain grew at the same rate as a normal strain. However, under anaerobic conditions, the menaquinone-deficient mutant grew slowly on glucose. Another mutant, deficient in both menaquinone

and ubiquinone, did not grow at all under the same conditions. It was shown by these workers that menaquinone was not involved in the aerobic metabolism of E. coli but was required for the anaerobic metabolism of reactions involved in pyrimidine biosynthesis, namely the oxidation of dihydro-orotate to orotate with fumarate as hydrogen acceptor.

SECTION 2.1 GENETIC AND BIOCHEMICAL CHARACTERISATION OF A MUTANT WHICH LACKS THE ABILITY TO FORM 4-HYDROXYBENZOATE.

INTRODUCTION.

In E. coli K-12, the first specific precursor in the biosynthesis of ubiquinone is 4-hydroxybenzoate and the formation of this compound from chorismate was initially shown by Gibson and Gibson (1962) using crude cell-free extracts of A. aerogenes 62-1. It was also demonstrated that 4-hydroxybenzoate could be formed chemically from chorismate (Gibson and Gibson 1962; Gibson 1964). Although the enzymic formation of 4-hydroxybenzoate appeared to be stimulated by NAD, the enzyme(s) concerned in the reaction had not been studied in any detail (Gibson and Gibson, 1964). The NAD stimulation has since been found to be due to the formation of 2,3-dihydroxybenzoate, which also arises from chorismate and has an absorption maximum at 252 nm, in the same region as 4-hydroxybenzoate (Young et al. 1967a).

A mutant strain of E. coli K-12 which is unable to carry out the first specific reaction of ubiquinone biosynthesis had been isolated by G.B. Cox and this section describes the genetic and biochemical characterization of this mutant strain. The original mutation was transferred into a new strain (AN244) which was used to characterize this mutation. In order to determine a ubiquinone-deficient strain, the lack of ability to grow on succinate as sole carbon source (and therefore to be a ubiquinone-deficient mutant),

depends on the rationale that follows. Since ubiquinone is implicated in membrane-bound electron transport processes (Crane et al. 1957), a mutant which lacks ubiquinone would not be able to grow on a particular carbon source if an essential step in its metabolism involves electron transport. Therefore ubiquinone-deficient strains might be found among mutants unable to grow on succinate, malate or lactate as sole carbon source (Cox, Gibson and Pittard, 1968). A mutant strain which lacks the ability to form 4-hydroxybenzoate would also be expected not to accumulate any of the known precursors of ubiquinone and not form ubiquinone unless supplied with 4-hydroxybenzoate.

MATERIALS AND METHODS.

Chemicals.

All chemicals used were obtained commercially and were of analytical grade, with the exception of chorismic acid which was prepared by the method of Gibson (1968).

Bacterial Strains.

A list of bacterial strains used in this section is shown in Table I. All the strains used were derived from E. coli K-12. Cultures were maintained on nutrient agar and sub-cultured monthly.

Media.

The minimal medium used was that described by Monod, Cohen-Bazire and Cohn (1951), and described as medium 56.

Medium 56.	K_2HPO_4	265g
	$NaH_2PO_4 . 2H_2O$	153g
	$MgSO_4 . 7H_2O$	5g
	$(NH_4)_2 SO_4$	50g

TABLE I. STRAINS OF E. COLI USED.

Strain	Relevant Genetic Loci[a]	Other Information
AN58	proA2, pheA1, tyrA4, tryp-401, argE3.	
AN244	ubiC401	Derived from AB259 after treatment with N-methyl-N'-nitrosoguanidine and subsequent recombination. (See text.)
AN245	proA2, argE3, pheA1, tyrA4.	Derived from AN58 by transduction (Huang and Gibson, 1970).
AN246	proA2, pheA1, tyrA4, metA$^-$	Derived from AN245 by transduction using P1Kc grown on arg$^+$ derivative of PA505MPE11 (Schwartz, 1966).
AN247	proA2, pheA1 tyrA4, ubiC401.	Derived from AN246 by transduction with P1Kc grown on AN244.
PA505MPE11	metA$^-$, argH$^-$	Schwartz (1966).
AT2246	leu-13, thr-13	Hfr PIO (Schwartz, 1966).
AB2830	aroC$^-$	Pittard and Wallace (1966).
AB3311	metB$^-$	Hfr Reeves
AN67	proA2, pheA1, tyrA4, tryp401, men$^-$	Derived from AN58 by transduction.

a The genetic nomenclature is that used by Taylor (1970), except for ubiC which is described in this thesis and men$^-$ which describes a gene concerned with menaquinone biosynthesis.

TABLE II. CONCENTRATION OF GROWTH FACTORS IN DEFINED MEDIA.

SUPPLEMENTS	FINAL CONCENTRATION
L-arginine	0.8 mM
L-phenylalanine	0.2 mM
L-tyrosine	0.1 mM
L-tryptophan	0.1 mM
L-methionine	0.2 mM
L-proline	1 mM
2,3-dihydroxybenzoate	10 µM
4-hydroxybenzoate	10 µM
Thiamine	0.02 µM
Succinate	20 mM
Glucose*	30 mM

* In anaerobic experiments glucose was used at a concentration of 30 mM, but in other experiments glucose was used in excess of 30 mM.

The salts were dissolved and made up to a volume of 5 l with distilled water. Chloroform (5 ml) was added to prevent bacterial growth in the stored medium. This solution was diluted 1:5 before use. Calcium and iron were added to the medium (1 ml/l) from stock solutions (1% $Ca(NO_3)_2$, 0.05% $FeSO_4.7H_2O$). Half-strength medium is referred to as $\frac{56}{2}$ and is medium 56 diluted 1:2 by vol. Sterile solutions of supplements were added to the media as required in the concentrations shown in Table II. The nutrient broth used for genetic experiments was that described by Luria and Burrous (1957) and consisted of the following:-

Difco Bacto Tryptone	10g
Difco Yeast Extract	5g
NaCl	10g
Distilled water	1ℓ

The pH was adjusted to 7.5 with NaOH. ½ broth was nutrient broth supplemented with 2.5×10^{-3}M $CaCl_2$. Solid medium was obtained by adding 2% (w/v) agar, in the case of minimal medium, and 1% (w/v) agar, in the case of nutrient medium.

Tests for Growth Response to 4-Hydroxybenzoate.

Growth responses of the various bacterial strains to 4-hydroxybenzoate were difficult to detect on a solid medium since low concentrations of 4-hydroxybenzoate are often found

as a contaminant in media. To prevent the formation of 4-hydroxybenzoate during the preparation of media, solutions of tyrosine were sterilized by filtration. Alternatively, 4-aminobenzoate at a final concentration of 50μM was added to the medium containing succinate as carbon source. Davis (1951) observed that concentrations of 4-aminobenzoate of the order of 5mM inhibited the growth of E. coli strains and that this inhibition of growth could be reversed by the addition of 4-hydroxybenzoate at a concentration of 50μM. The growth of the wild type strain was not visibly affected at the concentration of 4-aminobenzoate used. Growth responses of the multiple aromatic auxotroph AB2830 and the ubiC$^-$ strain AN247 to varying concentrations of 4-hydroxybenzoate, were carried out in a liquid medium supplemented with the required growth factors and succinate (20mM) as carbon source. The inoculum for the T-tubes was obtained by incubating the cells from a nutrient agar slope at 37^{o}C for 24 h in nutrient broth. These cells were concentrated by centrifugation and made up to 1 ml with minimal medium. T-tubes containing 10 ml of the liquid medium and supplements were inoculated with 0.1 ml of the concentrated suspension to give an initial population of 10^6 cells. The T-tubes were then shaken for 20 h at 37^{o}C and growth was measured using a Spekker colorimeter with a neutral density filter.

Genetic Techniques.

(a) Isolation of a streptomycin-resistant mutant.

The cells from a nutrient agar slope of strain AN244 were incubated overnight at 37°C in nutrient broth. A spontaneous streptomycin-resistant mutant of AN244 was isolated after spreading 0.1 ml of this culture onto nutrient agar plates containing 0.2 mg/ml of streptomycin sulphate and incubating overnight at 37°C.

(b) Soft agar overlay technique.

An aliquot of a sample (0.1 ml) was pipetted into a tube containing 3 ml of molten agar (1% w/v agar in medium $\frac{56)}{2}$ which had been stored at 45-50°C before use. The total contents of the tube were poured onto an agar plate, and then spread evenly.

(c) Conjugation technique.

The technique used for conjugation experiments was that described by Taylor and Thoman (1964). Experiments between Hfr male strains and F^- strains were designed to map new mutations i.e. to determine the approximate position of the genetic loci of a mutation in relation to other known markers. This was achieved by selecting recombinants on appropriate media. Male and female cells were grown in Luria broth to logarithmic phase ($\sim 10^8$ cells/ml). The male culture (1ml) was mixed gently with the female culture (9ml) for 5 min.

The time of entry of chromosomal markers was determined by sampling an aliquot (1ml) of the mixture at regular intervals, agitating on a Vortex mixer to separate the mating pairs, and selecting for recombinants using the soft agar overlay technique. Growth of parental cells was inhibited by the use of an appropriate defined medium or by the action of an antibiotic.

(d) Preparation of phage lysates.

Bacteria on which the generalized transducing bacteriophage P1Kc was to be prepared, were grown in Z broth to a cell density of about 10^9 cells/ml. The culture (1.5ml) was mixed with a phage P1 lysate (0.5ml - 10^9 p.f.u./ml) and aliquots (0.1ml) were overlaid onto Z plates using the soft agar overlay technique. After incubation at 37°C for 8 h, Z broth (2 ml) was added to each plate. Following a further incubation for approximately 16 h, the soft agar was removed from the plate, macerated to pulp, and then centrifuged to remove cell debris and agar before being filtered through a Millipore membrane filter. Preparations were stored at 4°C sterile, screw-cap Pyrex test tubes. The number of plaque forming units/ml of undiluted phage preparation was approximation 10^6 to 10^9.

(e) Transduction technique.

Transduction experiments using the generalized transducing bacteriophage P1Kc were performed according to the method of Pittard (1965). Bacteria to be transduced were grown overnight in Z broth (10 ml), centrifuged and resuspended in Z broth (1 ml). An aliquot of the cell suspension ($\sim 10^9$ cells) was mixed with appropriate volume of phage lysate, using an approximate phage to cell ratio of 1:1 in a total volume of 5 ml. After incubation at 37°C for 20 min, the cells were centrifuged and resuspended in minimal medium (1 ml). Diluted and undiluted aliquots were spread on plates of the appropriate selection media. Plates were incubated at 37°C for 2 days before examining the colonies of transductants that appeared.

Preparation of Cell-Free Extracts.

Cultures were grown in minimal media supplemented, as required, with the growth factors, as in Table I. Cells were harvested in early stationary phase of growth and were washed with cold 0.9% NaCl. Each gram of cells (wet weight) was suspended in 3 ml cold 0.05M Tris-Hcl buffer (pH 8.0) containing 10^{-3}M EDTA, and smashed in a French Press at 20,000 psi. The cell extracts were then centrifuged at 25,000 x g for 20 min at 4°C and the supernatants were used as the cell-free extracts.

Assay for 4-Hydroxybenzoate.

Chorismate lyase activity in strains AN246 and AN247 was determined using 0.5ml of cell-free extract (about 15 mg of protein) in a reaction mixture containing 2 µmoles of chorismate, 50 µmoles of L-tryptophan, 50 µmoles of Tris-HCl buffer (pH 8.0), and 1 µmole of EDTA in a final volume of 1 ml. L-tryptophan was added to repress anthranilate synthetase activity (Edwards et al., 1964) as this enzymic activity was normal in strains AN246 and AN247. Hence chorismate could be lost to this pathway. The reaction mixture and appropriate blank were incubated at 37°C for specific time intervals up to 3 h. The pH was lowered to 4.0 by the addition of 0.5 ml of 0.2M sodium acetate buffer (pH 4.0) and samples were refrigerated until required for extraction (pH 4.0 was chosen since little or no chorismate was extracted at this pH). Samples were extracted with 5 ml diethylether. The ether was dried over anhydrous sodium sulphate and the spectra were measured from 230 nm to 360 nm with a Model 15 Cary Spectrophotometer. 4-Hydroxybenzoate in ether has an absorption maximum at 252 nm (ε = 16,400). The spectra were always examined in detail, for in some experiments small amounts of 2,3-dihydroxybenzoate were formed. This compound also has a peak of absorption in the 250 nm region (ε = 7,200 at 252 nm). However, the formation of 2,3-dihydroxybenzoate in the reaction mixture could readily be observed

by the presence of a second peak of absorption at 320 nm (ε = 3,340). An appropriate correction was therefore made when necessary.

Determination of Ubiquinone.

For the estimation of ubiquinone content in cells, approximately 3 to 5 g cells (wet weight) were continuously extracted in a Soxhlet thimble with 100 ml acetone for 2 h. The acetone was removed by evaporation and the residue was then extracted with 50 ml of light petroleum ether (b.p. 40^{o} - 60^{o}C). This extract was transferred to another container, evaporated to a small volume (1-2 ml) and then chromatographed on thick layer silica gel plates in a mixture of light petroleum ether (b.p. 40^{o} - 60^{o}C) - chloroform (3:7 by vol.) as described by Cox et al. (1968). The yellow bands of ubiquinone were removed from the plates, eluted from the silica gel with ether and the ultraviolet spectra recorded using a Model 15 Cary spectrophotometer. The concentration of ubiquinone was calculated using a molar extinction coefficient of 14,956 (272 nm).

Test for the Presence of 4-Hydroxybenzoate in Culture Supernatants.

Cultures of the various strains were grown in 1 l batches of a glucose mineral salts medium supplemented with

the various growth factors as required - see Table I. Cultures were grown to a cell density of about 10^9 cells/ml as determined by measuring the turbidity in a Klett-Summerson colorimeter. The cells were removed by centrifugation at 17,500 x g for 15 min. The supernatants (100ml) were acidified to pH 4.0 with dilute hydrochloric acid before extracting with 3 vol. ether. The ether extract was then extracted with 0.1M NaOH and the aqueous layer was then acidified and re-extracted with ether. The final ether extract was dried over anhydrous sodium sulphate before evaporating to a small volume and applying to thin layer plates of silica gel. The plates were developed in chloroform-methanol-acetic acid (60:40:0.5 by vol.).

Protein Determination.

Protein was estimated using the Folin reagent as described by Lowry et al. (1951), and bovine serum albumin (Pentex) as a standard.

RESULTS.

Isolation of a Strain Carrying a Mutation Affecting 4-Hydroxybenzoate Synthesis.

During a search for strains of E. coli unable to form ubiquinone (Cox, Gibson, Pittard, 1968), a new strain was isolated. It would not grow with DL-lactate as sole source of carbon, but would grow on glucose (G.B.Cox, personal communication). This strain could not form ubiquinone when grown on a glucose medium, but would do so if 4-hydroxybenzoate was included in the medium. The conclusion drawn was that the reaction affected in ubiquinone biosynthesis was the conversion of chorisimate to 4-hydroxybenzoate and the gene concerned was designated ubiC. However, the ubiC$^-$ strain did not grow when the medium contained 4-hydroxybenzoate and either lactate or succinate. The possibility that there were other mutations affecting ubiquinone biosynthesis had therefore to be considered. Assuming there were two mutations, an interrupted mating experiment with the ubi$^-$ mutant strain (female) and a wild type Hfr was carried out in order to separate the two mutations. Recombinants were selected on the basis of their ability to grow on a succinate medium containing

4-hydroxybenzoate. One of these recombinants was isolated (strain AN244) and the mutation causing the requirement for 4-hydroxybenzoate was designated ubiC401 (G.B. Cox, personal communication).

Mapping of the ubiC Mutation.

Interrupted mating experiments were carried out to locate the approximate position of the ubiC mutation on the chromosome of E. coli. The results of experiments in which the female strain AN244 (ubiC$^-$) was mated with the Hfr strain AB3311 are shown in Figure 2.1. Selection for ubiC$^+$ recombinants was based on the ability of these recombinants to grow on succinate as sole carbon source. The ubiC$^+$ allele is transferred approximately 17 min after pair formation indicating a location at about minute 80 depending on the lag period. As strain AB3311 transfers its chromosome from minute 74 with a clockwise order of gene transfer, the ubiC gene is transferred as an early marker. The Hfr AT2246 has its origin of transfer near malB at minute 79 (Schwartz, 1966), and transfers its chromosome in the opposite direction to AB3311. Therefore, the strain AT2246 was used in an interrupted mating experiment. This strain did not transfer the ubiC gene as an early marker, although normal numbers of arg$^+$ recombinants were obtained in an interrupted mating experiment with the female strain JP58 (argE$^-$, ilvC$^-$), indicating that the ubiC gene was on the clockwise side of

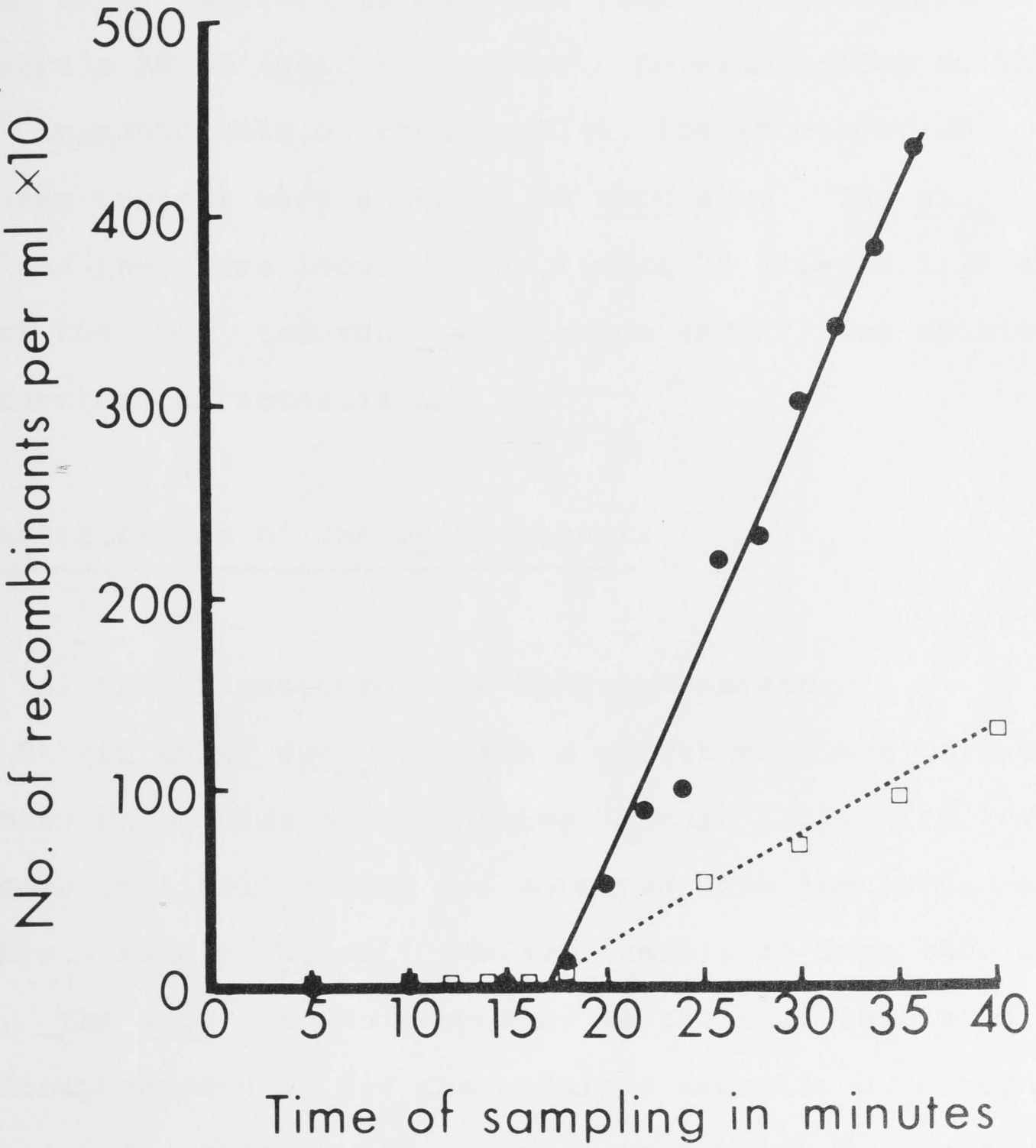


FIGURE 2.1 Kinetics of ubic$^+$ recombinant formation in two separate experiments during matings between the Hfr strain AB3311 and the F$^-$ strain AN244.

malB. A cotransduction test was then carried out with strain AN246 (derived as shown in Table I) as recipient and strain AN244 (ubiC$^-$) as donor. On examination of the metA$^+$ transductants of strain AN246, 11% (9 out of 80 colonies tested) were shown to be ubiC$^-$ also. The ubiC gene was therefore located near minute 79 (Figure 2.2) and one of the ubiC$^-$ transductant strains (AN247) was retained for further characterization.

Characterization of the ubiC$^-$ Mutant.

(a) Growth response to 4-hydroxybenzoate.

Strain AN247 (ubiC$^-$), gave a growth response to 4-hydroxybenzoate on succinate agar plates (Figure 2.3). The level of response in liquid medium was compared with that of an auxotrophic strain of E. coli (AB2830) unable to form chorismate. The ubiC$^-$ strain responded to the same levels of 4-hydroxybenzoate as did the multiple aromatic auxotroph (Figure 2.4), which is in contrast to some ubiA$^-$ strains which only respond to relatively high levels of 4-hydroxybenzoate (Cox et al., 1968).

(b) Accumulation of 4-hydroxybenzoate.

The presence of 4-hydroxybenzoate in the culture medium of ubiC$^-$ mutants could not be detected, whereas in culture fluids of wild type strains, 4-hydroxybenzoate is always formed. An excess of 4-hydroxybenzoate is excreted into

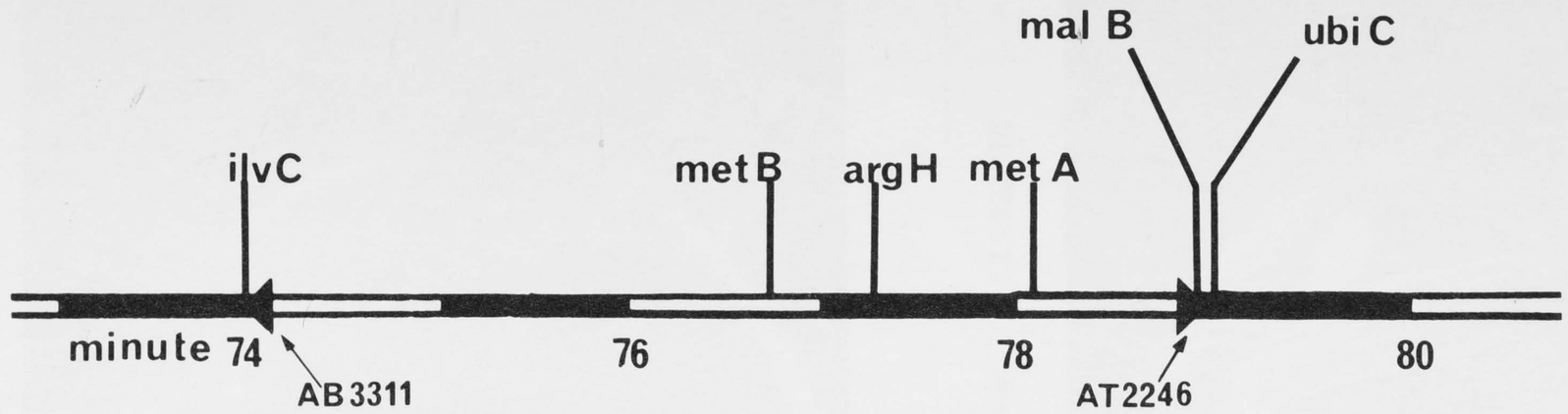


FIGURE 2.2 Genetic map showing the position of the ubiC gene and adjacent genes in the chromosome map of E. coli.

PLATE I. 4-HYDROXYBENZOATE ADDED.

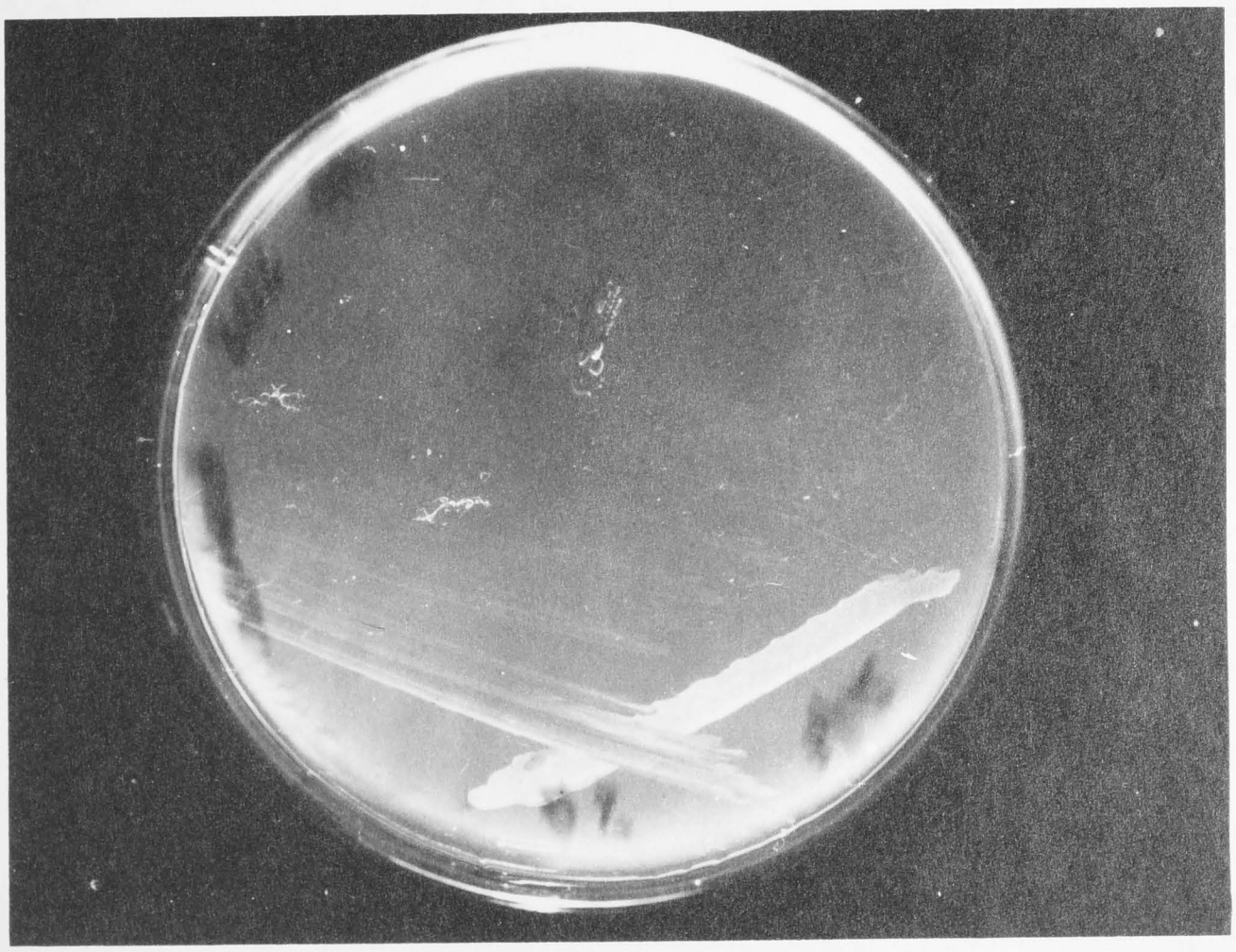


PLATE II. 4-HYDROXYBENZOATE ABSENT.

FIGURE 2.3 Growth response of strain AN247 (*ubiC*$^-$) to 4-hydroxybenzoate on succinate agar plates.

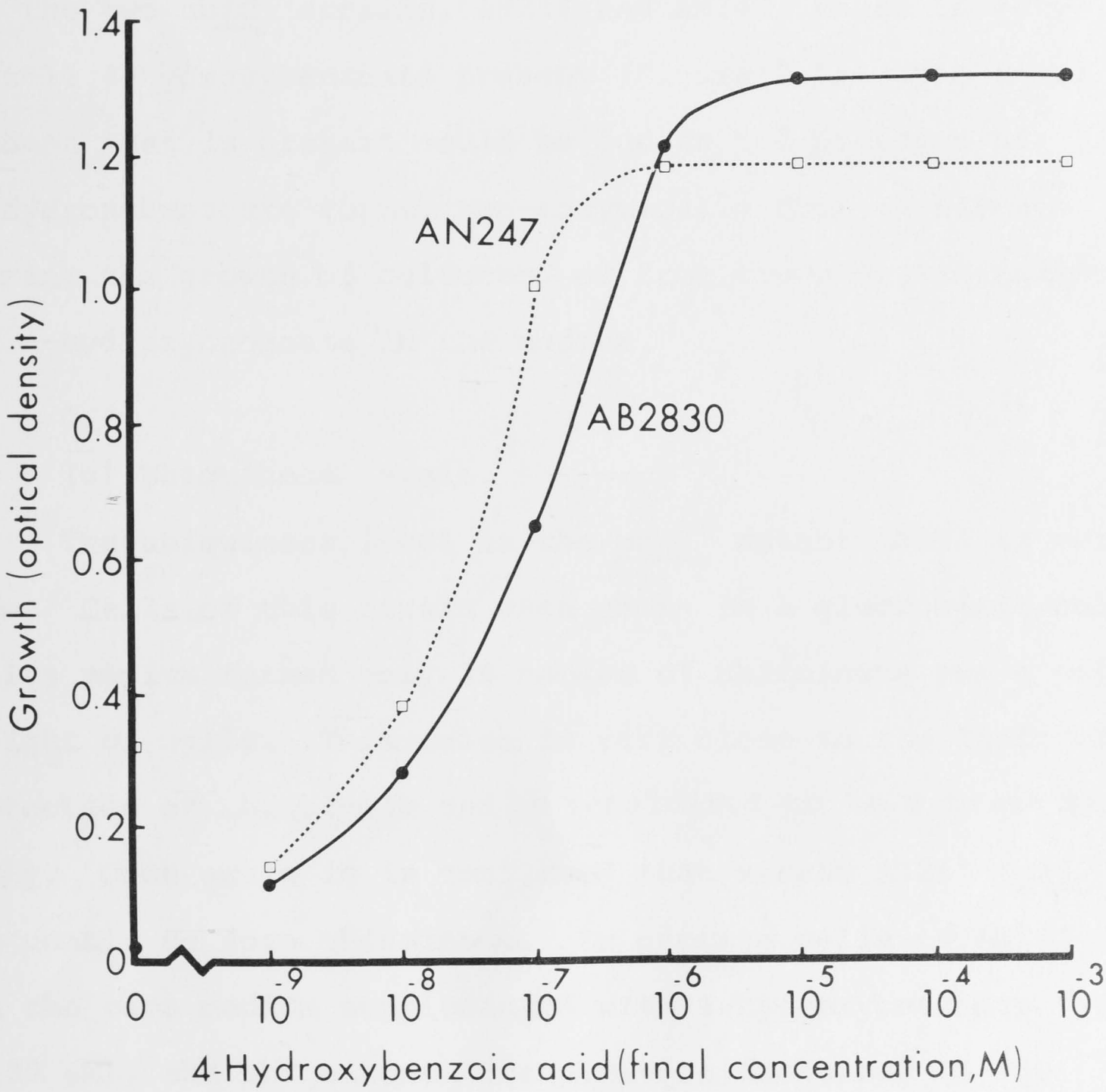


FIGURE 2.4 Growth responses of the multiple aromatic auxotroph AB2830 and the ubiC⁻ strain, AN247, to varying concentrations of 4-hydroxybenzoate in liquid medium containing succinate (20 mM) as carbon source.

the medium in the normal strain, AN246, whereas in the case of the two ubiC$^-$ strains, AN244 and AN247, there is very little 4-hydroxybenzoate present (Figure 2.5). The small amount that is present could be due to the presence of 4-hydroxybenzoate formed non-enzymically from chorismate during the growth of cultures, or from trace contamination of 4-hydroxybenzoate in the medium.

(c) Ubiquinone levels.

The ubiquinone level in the ubiC$^-$ mutant AN247 is very low. Cells of this strain when grown in a glucose-mineral salts medium formed only 20 nmoles of ubiquinone per g wet weight of cells. This value is very close to the limit of detection of ubiquinone and is considered to be a trace amount only. Once again it is confirmed that strain AN247 (ubiC$^-$) is unable to form ubiquinone. On growing cells of AN247 in the same medium supplemented with 4-hydroxybenzoate (100 μM), the ubiquinone concentration increases to 150 nmoles per g wet weight of cells, which is the normal level found in E. coli under the growth conditions used.

(d) Chorismate lyase activity.

Tests for chorismate lyase in the ubiC$^-$ transductant strain AN247 and the parent strain AN246 were carried out and the results are shown in Figure 2.6. In order to detect enzymic activities which convert chorismate along the minor pathways of aromatic metabolism, strains with

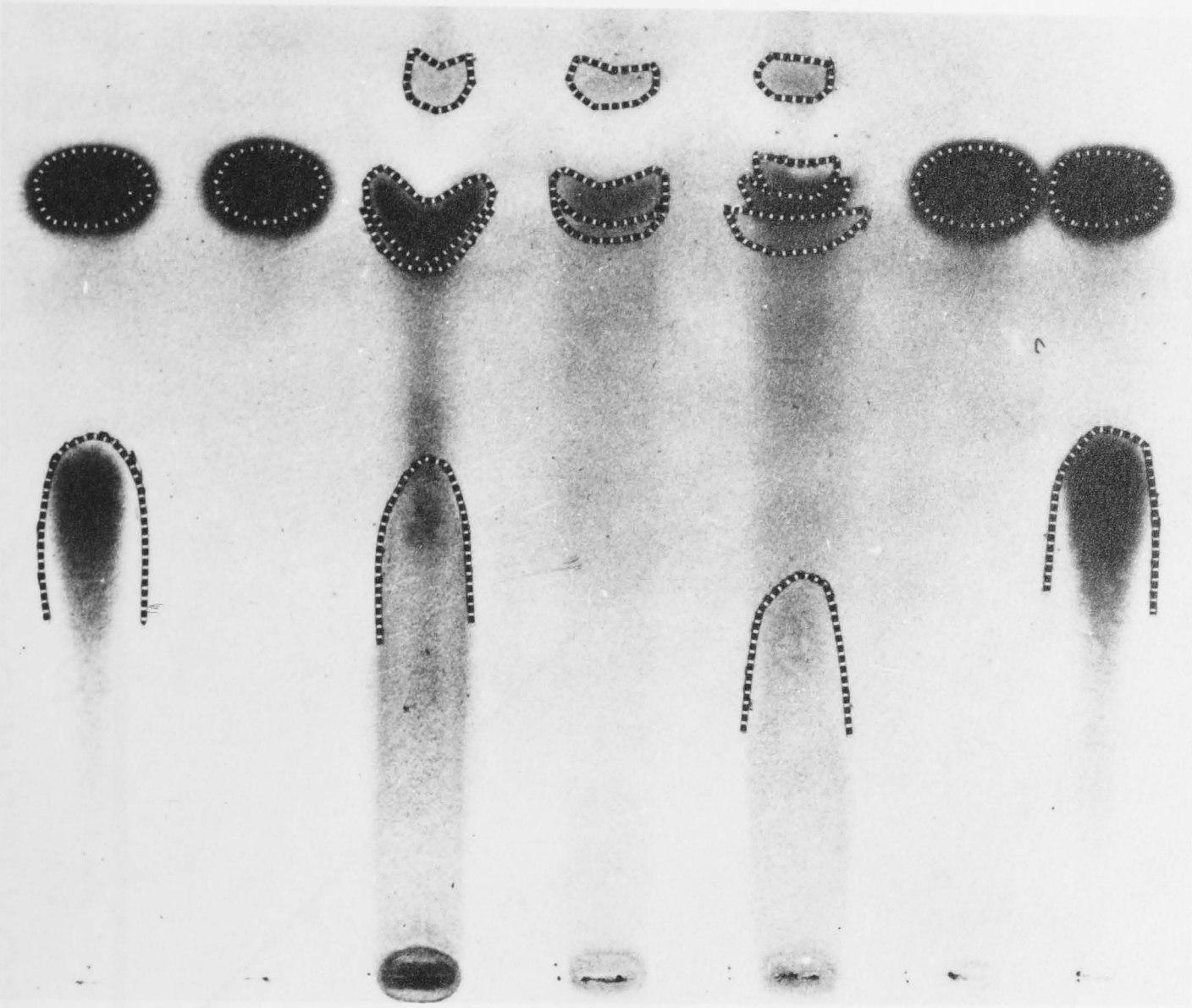


FIGURE 2.5 Chromatography of culture fluids of the normal strain of E. coli, AN246, and the two ubiC⁻ mutants, AN244 and AN247, to show the accumulation of 4-hydroxybenzoate. 4-Hydroxybenzoate is detected by spraying with p-nitraniline. The compounds are : A. unidentified; B. 4-hydroxybenzoate (colour - red-brown); C. 2,3 dihydroxy-benzoate (colour - purple).

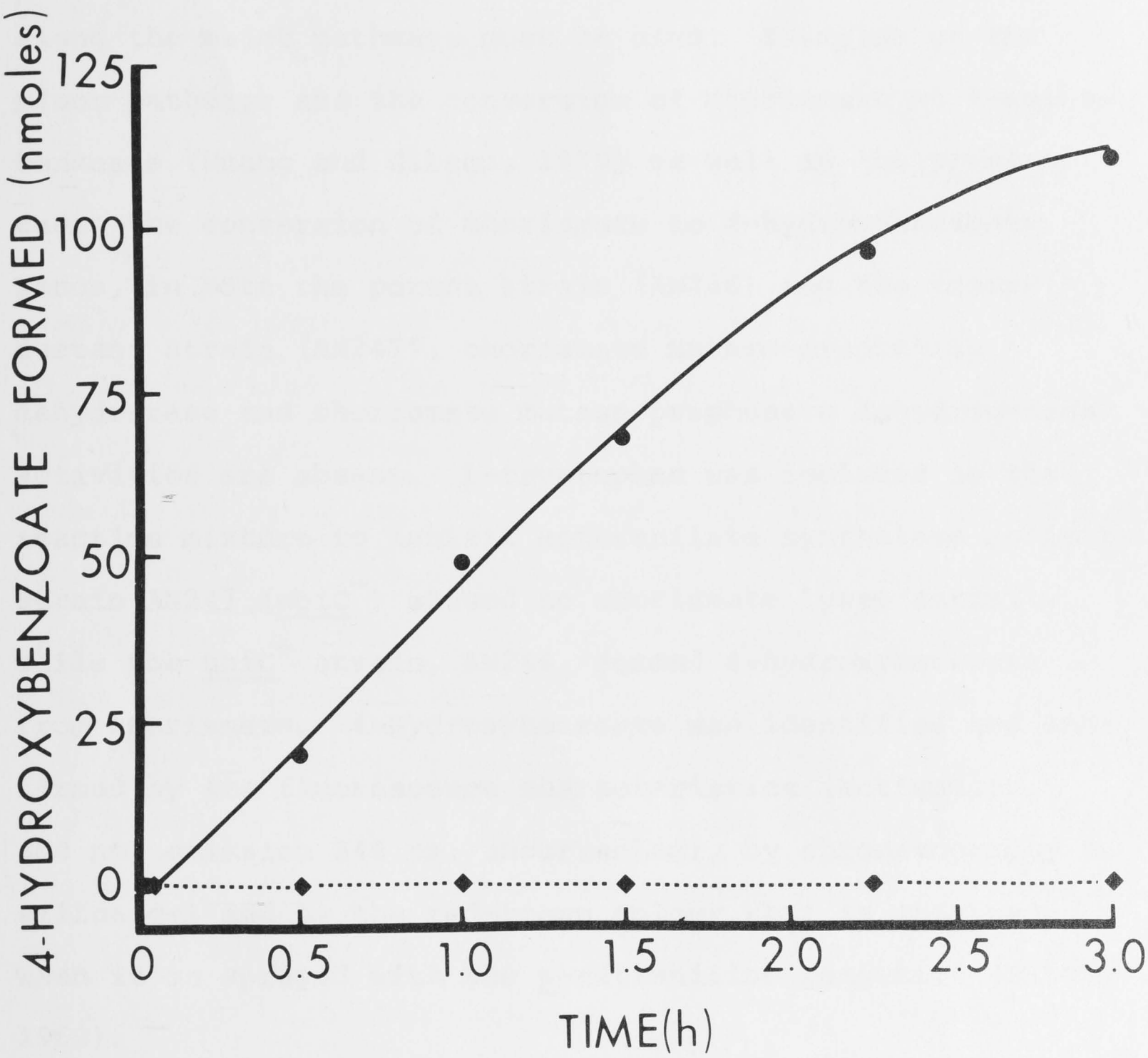


FIGURE 2.6 The formation of 4-hydroxybenzoate from chorismate by cell-free extracts of strain AN246 (wild type) ●——● and strain AN247 (ubiC⁻) ◆----◆ Conditions are those described in MATERIALS AND METHODS.

metabolic blocks preventing the metabolism of chorismate along the major pathways must be used. Examples of the minor pathways are the conversion of chorismate to 4-amino-benzoate (Huang and Gibson, 1970) as well as the present case, the conversion of chorismate to 4-hydroxybenzoate. Hence, in both the parent strain (AN246) and the trans-ductant strain (AN247), chorismate mutase-prephenate dehydratase and chorismate mutase-prephenate dehydrogenase activities are absent. L-tryptophan was included in the reaction mixture to inhibit anthranilate synthetase activity. Strain AN247 (*ubiC*$^-$) showed no chorismate lyase activity while the *ubiC*$^+$ strain, AN246, formed 4-hydroxybenzoate from chorismate. 4-Hydroxybenzoate was identified and con-firmed by its fluorescence characteristics (activation, 300 nm, emission 346 nm; uncorrected), by chromatography on silica gel and by the red-brown colour that is obtained when it is sprayed with the *p*-nitraniline reagent. (Smith, 1960).

DISCUSSION.

The ubiC gene was shown by interrupted mating experiments with the Hfr AB3311 and more accurately with transduction experiments, to be located at about minute 79 on the E. coli chromosome. The fact that the ubiC gene is not transferred as an early marker by strain AT2246 also supports this location on the chromosome. This is in the same region as the ubiA gene which codes for the enzyme catalysing the second specific reaction in ubiquinone biosynthesis, namely 4-hydroxybenzoate octaprenyltransferase (Young et al., 1972). The locations on the E, coli chromosome of seven genes concerned with ubiquinone biosynthesis have now been determined, and these are shown in Figure 2.7 (Cox, Gibson and Pittard, 1968; Cox et al., 1969; Young et al., 1971; Stroobant et al., 1972; Young et al., 1972;). While the ubiF, ubiG and ubiH genes are well scattered around the chromosome, there are two clusters of closely linked genes, namely the ubiB, ubiD, and ubiE genes and the ubiA and ubiC genes. The significance, if any, of the clustering has yet to be determined but it may be noted that both the ubiC and ubiA genes code for consecutive reactions, but this does not appear to apply to the other cluster of genes.

FIGURE 2.7 Genetic map of the E. coli chromosome showing the positions of the ubi genes. The scale on the genetic map is in minutes and is based on the map of the E. coli chromosome given by Taylor (1970).

The observations presented here indicate that a mutation in a gene designated ubiC, results in the loss of the ability to carry out the conversion of chorismate to 4-hydroxybenzoate. This conclusion is based on enzymic studies and on the requirement of 4-hydroxybenzoate for ubiquinone formation. Although a small amount of ubiquinone is formed by the ubiC$^-$ strain in the absence of added 4-hydroxybenzoate, this could be due to traces of 4-hydroxybenzoate formed from the non-enzymic break-down of chorismate, or formed from tyrosine during the growth of cultures.

SECTION 2.2 REGULATION OF CHORISMATE LYASE.

INTRODUCTION.

Few studies have been carried out on the regulation of the ubiquinone pathway. In this section mutant strains of E. coli have been studied with respect to the regulation of ubiquinone biosynthesis in whole cells. These studies have been limited to a consideration of effects of oxygenation on ubiquinone formation.

The mutant strains of E. coli used were of two types. Strain AN58 is a triple mutant (as described in Table I Section 2.1) and was used as the wild type strain. Strain AN67 is a triple mutant which lacks the ability to form menaquinone. Since ubiquinone and menaquinone are structurally related and are both derived from the branch-point compound, it was of interest to investigate the effects of the presence and absence of these compounds on chorismate lyase activity. This was done by growing cells under different conditions of aeration, since ubiquinone and menaquinone are present in aerobically grown cells of E. coli while under anaerobic conditions ubiquinone is absent and the menaquinone levels are increased (Lester and Crane, 1959; Polglase et al., 1966; Newton et al., 1971).

The effect of the level of 2-octaprenylphenol on chorismate lyase levels was also investigated. 2-Octaprenylphenol is only found in anaerobically grown cells of E. coli. By observing the 2-octaprenylphenol levels in normal and menaquinone-deficient strains when the cells were grown under anaerobic conditions, the comparison could easily be made.

By estimating the levels of a biosynthetic intermediate in the ubiquinone pathway and comparing it with the level of chorismate lyase present at the same stage of growth, the compound governing the regulation of this enzyme may be determined.

MATERIALS AND METHODS.

Chemicals.

All chemicals were obtained commercially and were of analytical grade with the exception of chorismic acid which was prepared by the method of Gibson (1968).

Bacterial Strains.

Strain AN58 and Strain AN67 (men^-) were used and are listed with their relevant genetic loci and other information in Table I, Section 2.1.

Media.

The minimal medium used was that described by Monod, Cohen-Bazire and Cohn (1951). Sterile solutions of the supplements were added to this medium as required in the concentrations shown in Table II, Section 2.1. Where cells were grown under anaerobic conditions, the medium for the inoculum consisted of Difco Casamino acids dissolved in 1 ℓ of distilled water to give a final concentration of 0.6% in 40 ℓ. Glucose was used as sole carbon source in all experiments.

Growth of Cells.

Cells were grown at 37°C in a New Brunswick Fermacell Fermenter. Under aerobic conditions, the rate of aeration was 2 cu. ft. per min and the speed of agitation was 300 revolutions per min. Under anaerobic conditions the Fermacell was supplied with nitrogen and carbon dioxide and pH control was used. Oxygen free nitrogen was obtained by passing the nitrogen gas through a furnace of activated cupric oxide pellets at 140°C. The inoculum for aerobic growth consisted of cells washed with distilled water from ten nutrient agar slopes which had been incubated overnight at 37°C. The inoculum for anaerobic growth consisted of cells washed from twenty nutrient agar slopes with the Casamino acid solution. The Casamino acid solution together with the cell suspension was poured into screw cap bottles which were then placed in anaerobic culture jars containing a 'cold' catalyst. The jars were partially evacuated, filled with hydrogen and incubated for approximately 30 h at 37°C. Cells were grown in minimal medium with supplements added as required, and glucose was the sole carbon source. Cells were harvested at early log phase, mid-log phase, stationary phase, and late stationary phase of growth. The growth curves for each organism were followed in order to determine the time of harvesting. Cells were harvested, centrifuged at 17,500 x g for 10 min and stored at -20°C until required for analysis.

Preparation of Cell-Free Extracts.

Cells were resuspended in 3 vol. of cold 0.1M Tris-HCl buffer (pH 8.0) containing 10^{-3} M EDTA, and smashed in a French Press at 20,000 psi. Cell debris was removed by centrifugation at 25,000 x g for 30 min giving the cell-free extract.

Assay Techniques.

(a) Determination of ubiquinone, menaquinone and 2-octaprenylphenol.

The method of estimating the concentrations of ubiquinone, menaquinone and 2-octaprenylphenol was the same as that used for the estimation of ubiquinone content in Section 2.1. Menaquinone and 2-octaprenylphenol were isolated from the same plates as ubiquinone. The yellow bands of ubiquinone and menaquinone were each eluted into diethyl ether. The position of 2-octaprenylphenol was detected by spraying a small section of the plate with the p-nitraniline spray (Cox et al., 1969), giving a dark red band running above ubiquinone. This area of the plate was then extracted into diethyl ether. The ubiquinone, menaquinone and 2-octaprenylphenol concentrations were determined from their ultraviolet spectra in ether recorded on a Model 15 Cary Spectrophotometer. A molar extinction value

of 19,189 at 248 nm, was used for menaquinone, while a molar extinction of 2,000 at 274 nm was used for 2-octaprenylphenol.

(b) Measurement of oxygen uptake.

Oxygen uptake by cell-free extracts was measured as described by Cox et al. (1970). A Titron oxygen electrode (Titron Instruments, Melbourne, Victoria) modified as described by Snoswell (1966) was used. The reaction mixture in a final volume of 2.5 ml contained 15 mM sodium-potassium phosphate buffer (pH 7.4), 1.9mM $MgCl_2$, 100 µl cell-free extract (3-5 mg protein) and 3 µ moles NADH.

(c) Estimation of chorismate lyase activity.

Chorismate lyase activity was determined in the same manner as described in Section 2.1., namely by estimating the amount of 4-hydroxybenzoate formed from cell-free extracts on a Model 15 Cary Spectrophotometer.

(d) Protein Determination.

Protein was determined in cell-free extracts using the Folin reagent as described by Lowry et al. (1951).

RESULTS.

Growth Curves and Growth Yields for the Wild-type Strain and the Menaquinone-Deficient Strain.

When comparing the aerobic growth curves for the normal strain AN58 and the menaquinone-deficient strain AN67, growing on a medium with glucose as sole source of carbon, there was little difference in the two curves. The mean generation time for both strains was 1 h and the growth yields were comparable (Figures 2.8 and 2.9). However, when both strains were grown under anaerobic conditions, there was a marked difference in both the growth curves and growth yields (Figures 2.10 and 2.11). A linear curve was obtained for strain AN67 (men^-) indicating a possible requirement for growth. Also the mean generation time for strain AN67 (men^-) was 3 h whereas for strain AN58 (wild type) it was 1 h. Slightly lower yields were obtained for the normal strain when grown under anaerobic conditions. However the yield of the menaquinone-deficient strain dropped to one third under these same conditions. This made the comparison of the two strains under anaerobic conditions more difficult. To estimate the quinone content and chorismate lyase activity, five or six samples were usually taken during

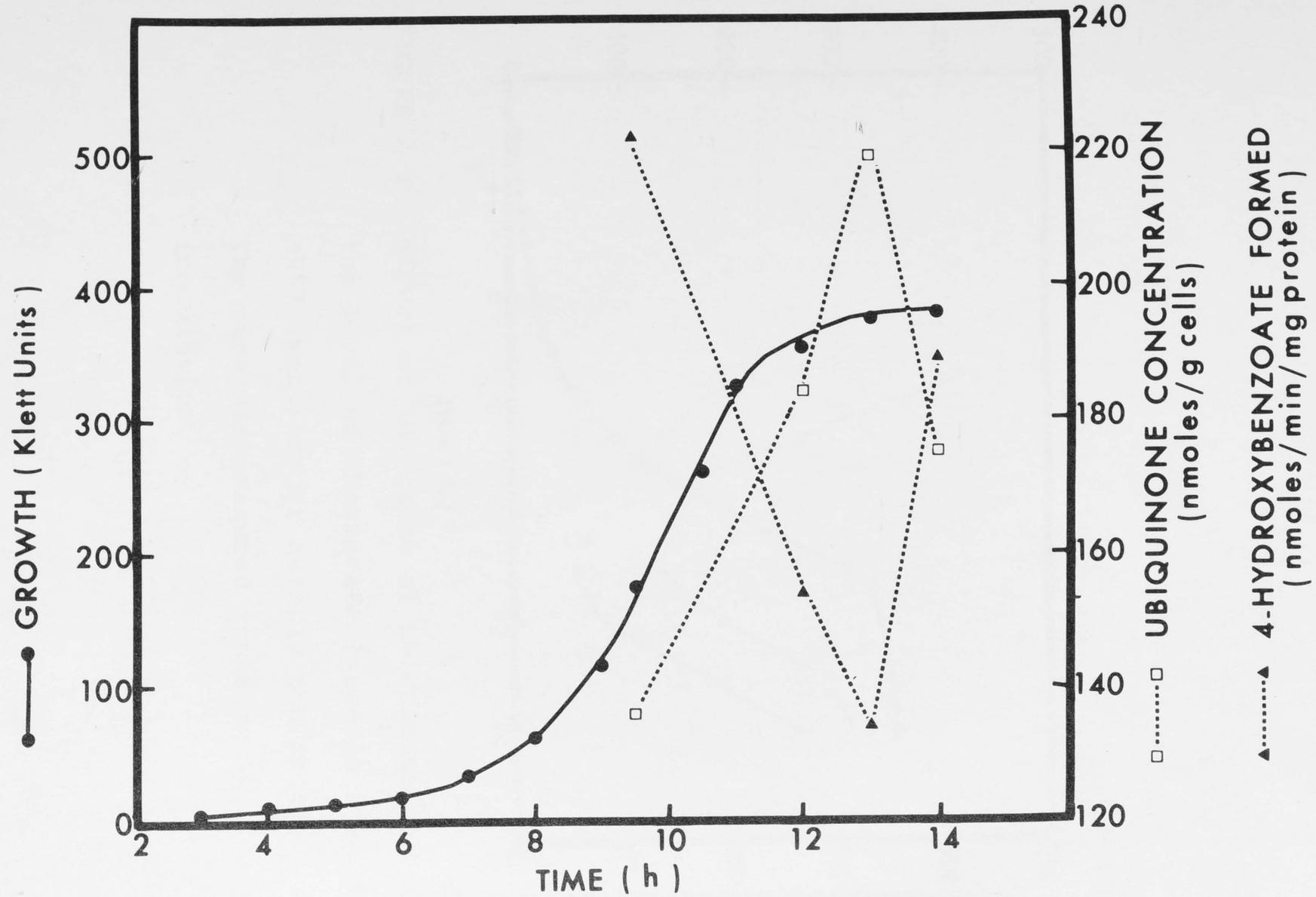


FIGURE 2.8 Effect of the level of ubiquinone on the level of chorismate lyase in strain AN58 under aerobic conditions. The time was measured starting at inoculation.

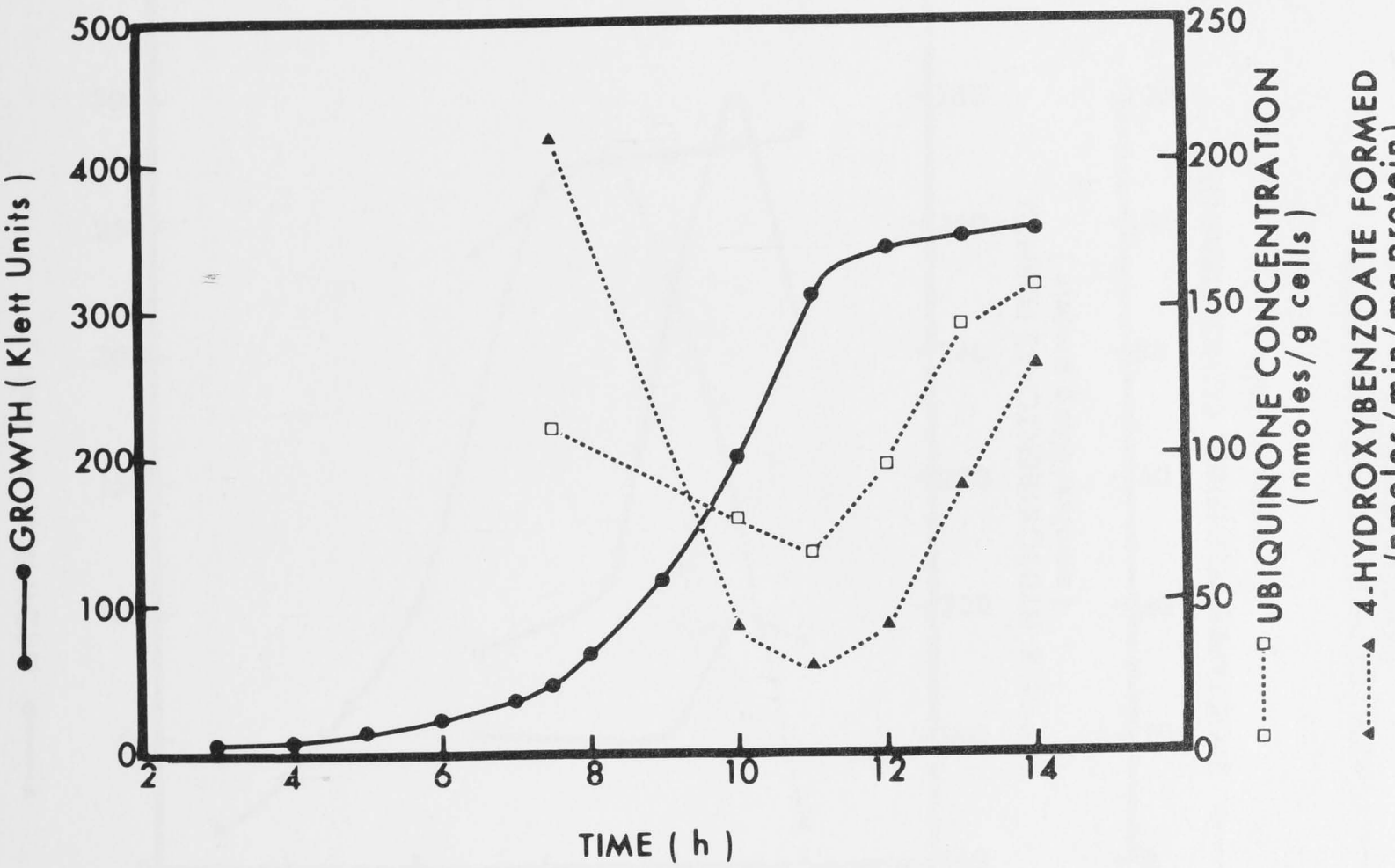


FIGURE 2.9 Effect of the level of ubiquinone on the level of chorismate lyase in strain AN67 (men$^-$) under aerobic conditions. The time was measured starting at inoculation.

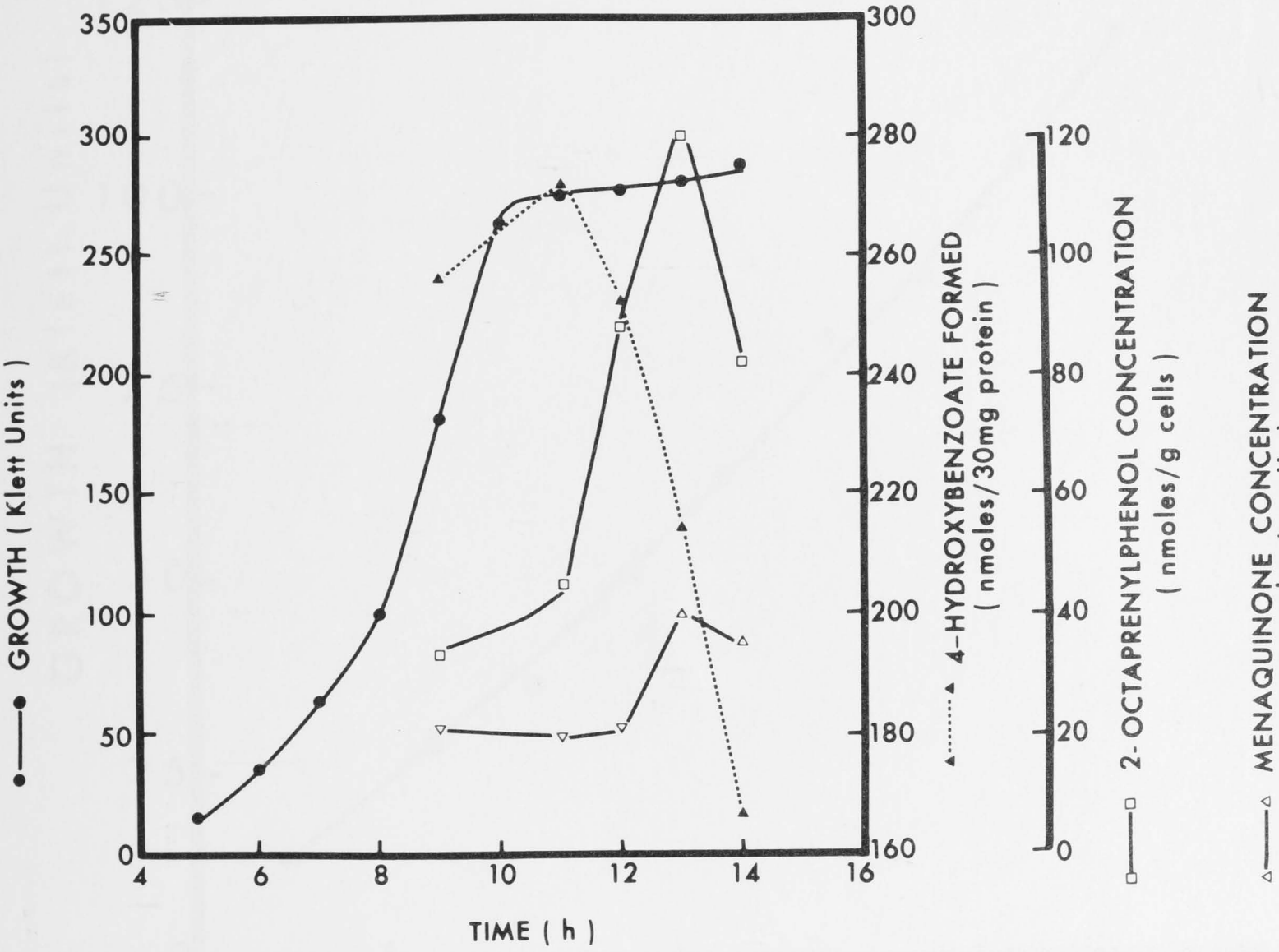


FIGURE 2.10 Effect of the levels of menaquinone and 2-octaprenylphenol on the level of chorismate lyase in strain AN58 under anaerobic conditions. No ubiquinone could be detected under these conditions. The time was measured starting at inoculation.

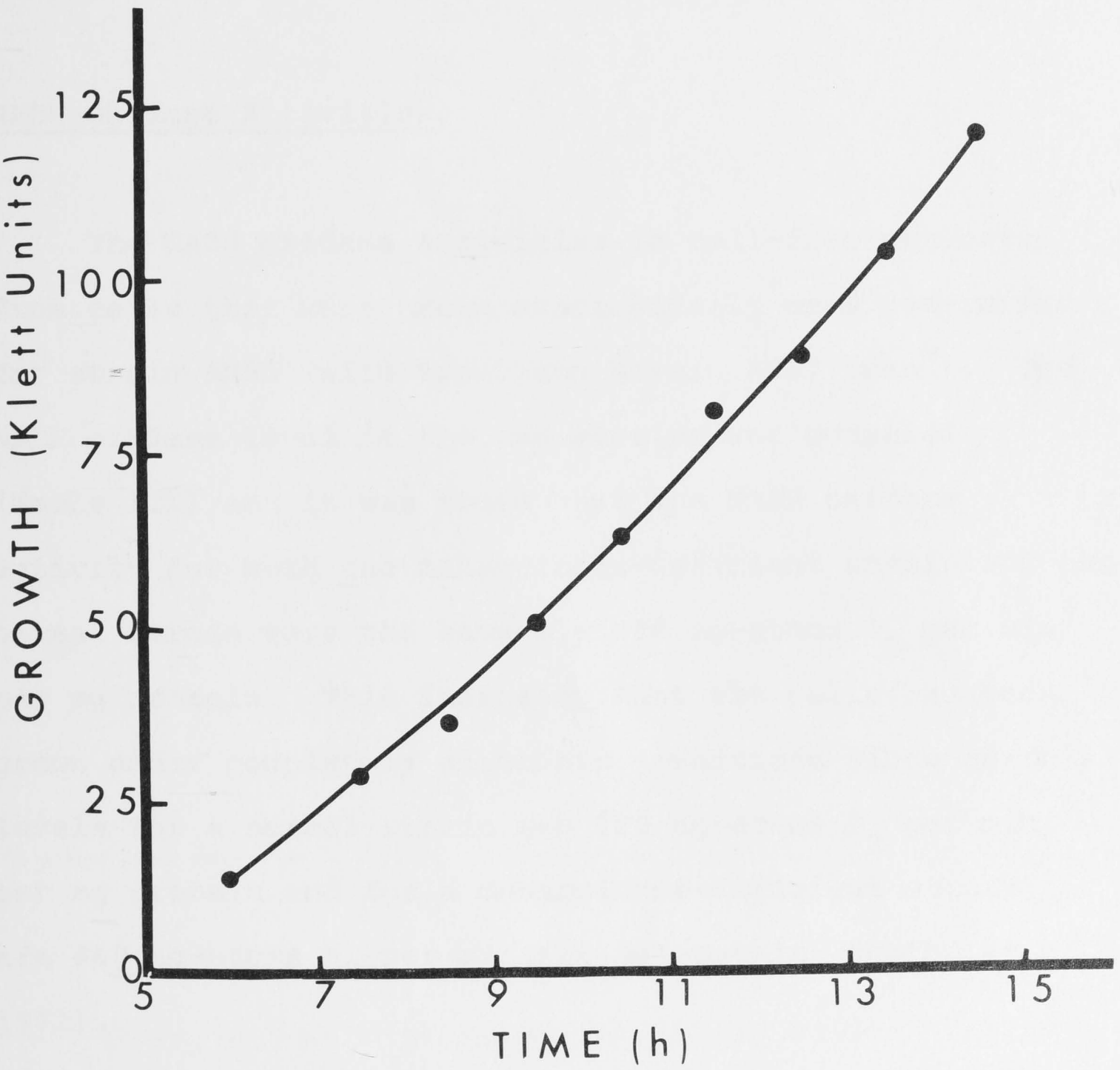


FIGURE 2.11 Growth curve for strain AN67 (men^-) grown under anaerobic conditions.

the growth of the normal strain. However, it was only possible to take two samples with the menaquinone-deficient strain due to the low growth yield.

NADH Oxidase Activities.

The NADH oxidase activities in cell-free extracts from cells that were grown anaerobically were determined for strain AN58 (wild type) and strain AN67 (men^-). The NADH oxidase level in the two strains was compared (Table III) and it was found that the NADH oxidase activity for both the menaquinone-deficient strain and the normal strain were the same i.e. 90 ng-atoms 0_2 per min per mg protein. This indicated that the cells had been grown under completely anaerobic conditions since aerobic levels for a normal strain are 780 ng-atoms 0_2 per min per mg protein and for a menaquinone-deficient strain are 860 ng-atoms 0_2 per min per mg protein (Newton et al. 1971).

Effect of Ubiquinone on Chorismate Lyase Activity under Aerobic Conditions.

The concentration of ubiquinone was estimated together with chorismate lyase activity in both strains at various stages during the growth cycle. Under aerobic conditions,

TABLE III. NADH Oxidase Activities in Cell-Free Extracts of Two Strains AN58 andAN67 Under Anaerobic Conditions.

Klett Units	O_2 uptake for Strain AN58 (wild type) (ng-atoms per min per mg protein)	Klett Units	O_2 uptake for Strain AN67 (men^-) (ng-atoms per min per mg protein)
182	87	70	100
275	62	120	94
278	46		
282	33		
288	35		

Rates of oxygen uptake were measured with an oxygen electrode as described in Materials and Methods (this section). The endogenous oxygen uptake (about 10 ng-atoms of O_2 per min per 100 µl) has been subtracted.

strain AN58 (wild type) showed a maximum concentration of ubiquinone with a corresponding minimum concentration of chorismate lyase activity; the amount of 4-hydroxybenzoate formed is an indication of the level of chorismate lyase activity present in the cell at any particular stage of growth (Figure 2.8). Strain AN67 (men^-), the quinone deficient strain, showed a pattern quite the reverse under aerobic conditions (Figure 2.9). Both the concentration of ubiquinone and the level of chorismate lyase activity were at a minimum during stationary phase of growth. If the ratio of 4-hydroxybenzoate concentration to ubiquinone concentration for strain AN58 (wild type) and strain AN67 (men^-) is calculated (Table IV), then for a given concentration of ubiquinone, the level of chorismate lyase activity decreases in the early stages of growth and then rises again toward the end of the growth cycle.

Effect of Ubiquinone and 2-Octaprenylphenol on Chorismate Lyase Activity under Anaerobic Conditions.

The most notable effect of ubiquinone on growth is observed under anaerobic conditions. When strain AN58 (wild type) is grown under anaerobic conditions, the level of chorismate lyase activity rises to a maximum at early stationary phase of growth and then decreases markedly in late stationary phase of growth (Figure 2.10). This is the opposite effect to strain AN58 (wild type) when

TABLE IV. Ratio of 4-Hydroxybenzoate Concentration to Ubiquinone Concentration.

Klett Units	Strain AN58 (wild type) Aerobically grown.	Klett Units	Strain AN67 (men^-) Aerobically grown.
175	1.632	45	1.90
353	0.837	200	0.55
375	0.612	310	0.43
380	1.08	340	0.44
		350	0.62
		355	0.835

grown under aerobic conditions (Figure 2.8). The absence of ubiquinone is noted under anaerobic conditions, whereas the concentration of menaquinone is relatively constant under these same growth conditions. However, the level of 2-octaprenylphenol for strain AN58 (wild type) rises to a maximum at a later stage in the growth cycle (late stationary phase) compared to the level of chorismate lyase activity (Figure 2.10). When the ratio of 4-hydroxy-benzoate to 2-octaprenylphenol concentration is calculated, a sharp decrease is observed in the level of chorismate lyase activity and this is followed by a constant level of activity (Table V).

When the cells of the strain AN67 (men^-) are grown under anaerobic conditions, ubiquinone is not usually found. If ubiquinone is present, the level is below the accuracy of detection. Although the results for strain AN67 (men^-) are too few to make a definite statement about any trend, they are nevertheless not unlike the results obtained for strain AN58 (wild type) grown under anaerobic conditions. The levels of 4-hydroxybenzoate and 2-octaprenylphenol in strain AN67 (men^-) at the same growth stage are comparable with the levels found in AN58 (wild type) grown under anaerobic conditions (Table VI).

TABLE V. Ratio of 4-Hydroxybenzoate Concentration to Ubiquinone Concentration.

Klett Units	Strain AN58 (wild type) Anaerobically grown.	Klett Units	Strain AN67 (Men-) Anaerobically grown.
182	7.7	70	6.9
275	6.0	120	6.4
278	2.9		
282	2.0		
288	2.0		

TABLE VI. Strain AN67 (men$^-$) Grown Under Anaerobic Conditions.

Klett Units	4-Hydroxybenzoate concentration (nmoles/30 mg protein)	2-Octaprenylphenol concentration (nmoles/g cells)
70	249	36
120	293	46

DISCUSSION.

The results obtained from the regulation studies on whole cells in two strains of E. coli indicate that ubiquinone is not involved in the regulation or control of chorismate lyase activity. This study has been carried out with two strains, one of which is menaquinone deficient and one of which is normal. The effects observed on the level of chorismate lyase activity in both strains under both aerobic and anaerobic conditions indicate that ubiquinone does not regulate the conversion of chorismate to 4-hydroxybenzoate and that the level of chorismate lyase activity is independent of the ubiquinone concentration at any given time (Figure 2.8, 2.9 and 2.10).

The level of chorismate lyase activity in strain AN58 (wild type) when grown under anaerobic conditions, initially rises then falls rapidly, and the increase in the 2-octaprenylphenol concentration is coincident with this fall- the peak of the 2-octaprenylphenol concentration is displaced to the right which could be indicative of its synthesis at a later stage in the ubiquinone pathway (Figure 2.10).

The rate of 4-hydroxybenzoate formation is steady until the enzymic reaction is regulated. Similarly the rate of 2-octaprenylphenol formation remains steady and is governed

by the supply of 4-hydroxybenzoate. Therefore, as the concentration of 2-octaprenylphenol builds up, there is a corresponding depletion in the concentration of 4-hydroxybenzoate. If there is to be any regulation in the level of chorismate lyase activity, then a point would be reached where at a certain concentration of 2-octaprenylphenol the first reaction in the ubiquinone pathway would be slowed down to a steady rate of production of 4-hydroxybenzoate i.e. the ratio of 4-hydroxybenzoate concentration to 2-octaprenylphenol concentration would stabilise at this stage.

The ratio for strain AN58 (wild type) grown under anaerobic conditions (as seen in Table V) clearly takes this trend unlike the ratio of the concentration of 4-hydroxybenzoate to ubiquinone concentration for the same strain under aerobic conditions. Therefore it may be concluded that 2-octaprenylphenol or one of the preceding compounds regulates the level of chorismate lyase activity.

A further point which suggests that 2-octaprenylphenol is regulating the level of chorismate lyase activity is that under aerobic conditions, when 2-octaprenylphenol is absent, the level of chorismate lyase activity increases in both strains (Figures 2.8 and 2.9) in stationary phase indicating that no regulation is taking place. Under anaerobic conditions when 2-octaprenylphenol is present, the level of chorismate lyase activity in strain AN58

(wild type) drops markedly in the stationary phase of growth and does not rise again even in late stationary phase of growth (Figure 2.10).

SECTION 3.1 PURIFICATION OF CHORISMATE LYASE.

INTRODUCTION.

The only previous studies on chorismate lyase were made by Gibson and Gibson (1962, 1964) who were able to detect enzymic activity in crude cell-free extracts of A. aerogenes 62-1. These authors did not undertake any further investigations of the properties of the enzyme. It was therefore of interest to further characterize chorismate lyase, especially as nothing was known about the properties of the enzymes responsible for the biosynthesis of ubiquinone. Efforts have been directed towards the development of a suitable procedure for the purification of the enzyme from extracts of a mutant strain of E. coli K-12 which had been blocked in the metabolic pathways that lead to tyrosine, phenylalanine, and tryptophan. Since the enzymic activity, as measured in crude extracts, was very low, the purification of the enzyme proved to be difficult. Nevertheless it was considered worthwhile devoting a great deal of time to this project in order to gain for the first time information about an enzyme involved in the synthesis of a vitamin. In particular, it was of interest to determine if the low enzymic activity of crude extracts was due to the low concentration of the enzyme within the cell and/or to its low turnover number.

MATERIALS AND METHODS.

Chemicals.

All chemicals used were obtained commercially and were of analytical grade. No further treatment was made to the chemicals unless stated otherwise. Chorismic acid was prepared by the method of Gibson (1968).

Microorganisms.

The organism used as the source of chorismate lyase was E. coli AN58 (Section 2.1 - Table I) isolated by M. Huang. This organism is a suitable choice of bacterium as it is a strain carrying three mutations affecting the metabolism of chorismate towards the three major end-products.

Growth Conditions for Strain AN58.

From the work carried out on the regulation of chorismate lyase in whole cells (Section 2.2), it was established that the activity of the enzyme in this organism, reaches a maximum value in early stationary phase of growth. The cells from

strain AN58 were therefore harvested at this point. The conditions for growing the cells and the preparation of cell-free extracts were the same as those described in Section 2.2.

Storage of Cells.

Cells were obtained in batches of 170 g from each run in the Fermacell fermenter (40 l). They were stored frozen as a paste at -20°C. The activity of the enzyme remained stable for several months when stored in this manner.

Determination of Chorismate Lyase Activity.

The activity of the enzyme in various column fractions during the purification procedure was measured spectrophotometrically by estimating the amount of 4-hydroxybenzoate formed from chorismate after incubating the reaction mixture for 2 h at 37°C (Section 2.1).

Units of Enzymic Activity.

One unit of chorismate lyase activity is defined as that amount of enzyme which produces 1.0 nmole of product per min at 37°C. Specific activity is defined as the number of units per min per mg of protein.

Assays for Protein.

Protein was determined in crude cell-free extracts as described by Lowry et al. (1951), using bovine serum albumin as a standard, and in column fractions by measuring the absorption at 280 nm with a Gilford 240 spectrophotometer.

Concentration of Protein.

Protein solutions were concentrated by ultrafiltration using a Diaflo apparatus (Amicon Co.) in conjunction with a UM-2 membrane which has a molecular weight exclusion limit of 1,000. The pressure was maintained between 40 and 60 lb/inch^2 with nitrogen gas.

Fractionation of Chorismate Lyase by High Speed Centrifugation.

In order to determine whether the enzyme was present in the soluble part of the cell, or was attached to the membrane, high speed ultracentrifugation studies were carried out on crude cell-free extracts of strain AN58 as described by Jones and Redfearn (1966). The crude cell-free extract was centrifuged at 35,000 x g for 30 min in a Beckman Model L ultracentrifuge to sediment the large particles. The turbid supernatant was further centrifuged

at 105,000 x g for 90 min to yield the small particles. The remaining yellow supernatant is referred to as the high speed supernatant and is equivalent to the soluble fraction of the cell extract. The small and large particle fractions were each resuspended in 0.05 M Tris-HCl buffer (pH 8.0) containing 10^{-3} M EDTA. These fractions, as well as the final supernatant fraction, were assayed for enzymic activity.

Treatment of Protein with Protamine Sulphate.

The amount of protamine sulphate required to completely precipitate the nucleic acid present in the crude extract was determined by titrating several aliquots of cell-free extract (5 ml) with increasing amounts of a 2% (w/v) protamine sulphate solution; the protamine sulphate was dissolved in 0.1M Tris-HCl buffer (pH 8.0) containing 10^{-3} M EDTA. The precipitates which formed were removed by centrifugation and each supernatant was tested with a concentrated solution of ribosenucleic acid (10 mg/ml) to detect excess protamine sulphate. The amount of protamine sulphate required for each preparation of cell-free extract was determined by such a titration.

Fractionation of Protein Using Ammonium Sulphate.

The weight of finally ground ammonium sulphate required to give the desired saturation was calculated using the equation of Kunitz (1952) viz;-

$$X = 53.3 \frac{(S_2 - S_1)}{1 - 0.3S_2}$$

where X represents the number of g of solid ammonium sulphate to be added to 100 ml of solution of saturation S_1 in order to change it to saturation S_2.

RESULTS.

Development of the Purification Procedure - Preliminary Experiments.

Chorismate lyase was found to be a soluble enzyme as judged by the fact that all the enzymic activity was located in the high-speed supernatant fraction (Table I). Thus chorismate lyase differs from two other enzymes which are involved in the ubiquinone pathway and which have been shown by Young et al. (1972) to be membrane bound.

(a) Protamine sulphate treatment.

The treatment of the cell-free extract with 2% (w/v) protamine sulphate (Figure 3.1) indicates that little activity is lost on the addition of increasing amounts of protamine sulphate. The amount of protamine sulphate required was determined for each batch of cells, since the protein content of the supernatant was variable and different batches of protamine sulphate also varied slightly.

TABLE I. Intracellular Distribution of Chorismate Lyase Activity in Subcellular Fractions of E. coli AN58.

Fraction	Chorismate lyase Activity ΔE_{252} per h
Cell-free extract	0.164
Large particles	0
Small particles	0
Supernatant	0.167

Cell extracts were fractionated as described by Jones and Redfearn (1966) in MATERIALS AND METHODS this section.

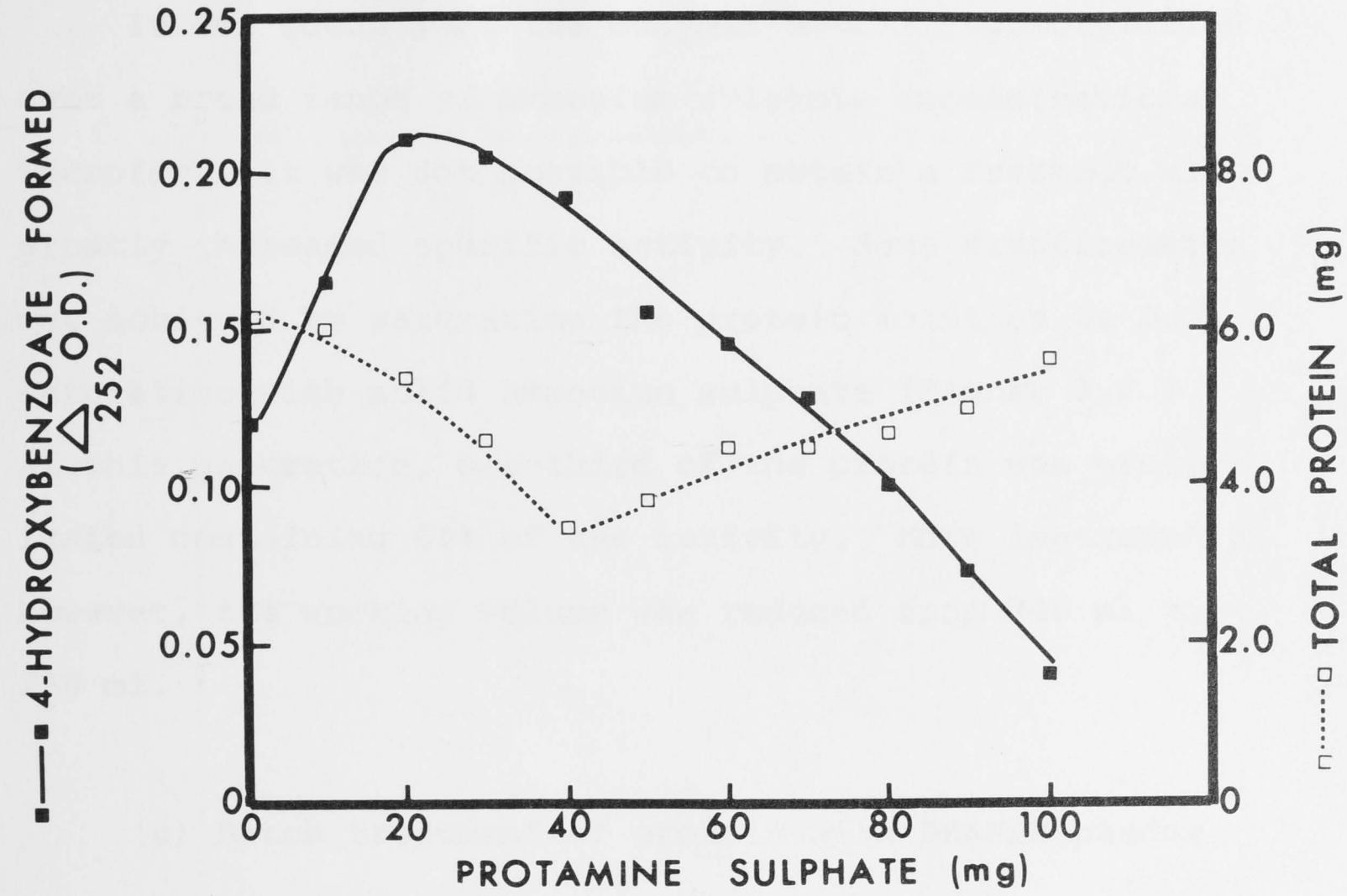


FIGURE 3.1 The effect of protamine sulphate on the activity of chorismate lyase. Each sample contained 5 ml of a cell-free extract of strain AN58. A 2% (w/v) solution of protamine sulphate was added to each sample of cell-free extract in amounts ranging from 10 to 100 mg, and then assayed for enzymic activity as described in MATERIALS AND METHODS.

(b) Ammonium sulphate fractionation.

It was found that the enzymic activity precipitated over a broad range of ammonium sulphate concentrations. Therefore, it was not possible to obtain a fraction with greatly increased specific activity. Some fractionation was achieved by saturating the protein solution to 0.5 saturation with solid ammonium sulphate (Figure 3.2.). At this saturation, one-third of the protein was precipitated containing 64% of the activity. More importantly however, the working volume was reduced from 700 ml to 150 ml.

(c) Batch treatment of protein with DEAE-Sephadex.

The concentrated protein solution from the previous step was dialysed overnight against 0.02M Tris-HCl buffer (pH 8.0) containing 10^{-3}M EDTA, and then applied to a pad of DEAE-Sephadex that had been previously equilibrated with the same buffer. After washing the pad with the same buffer, the molarity of the buffer was increased to 0.1M Tris (pH 7.0) and then to 0.2M Tris (pH 7.0). Pilot tests were carried out to determine which of the two buffer concentrations would elute the enzymic activity better, and also to determine whether the drop in pH would decrease the binding of the enzyme to the DEAE-sephadex.

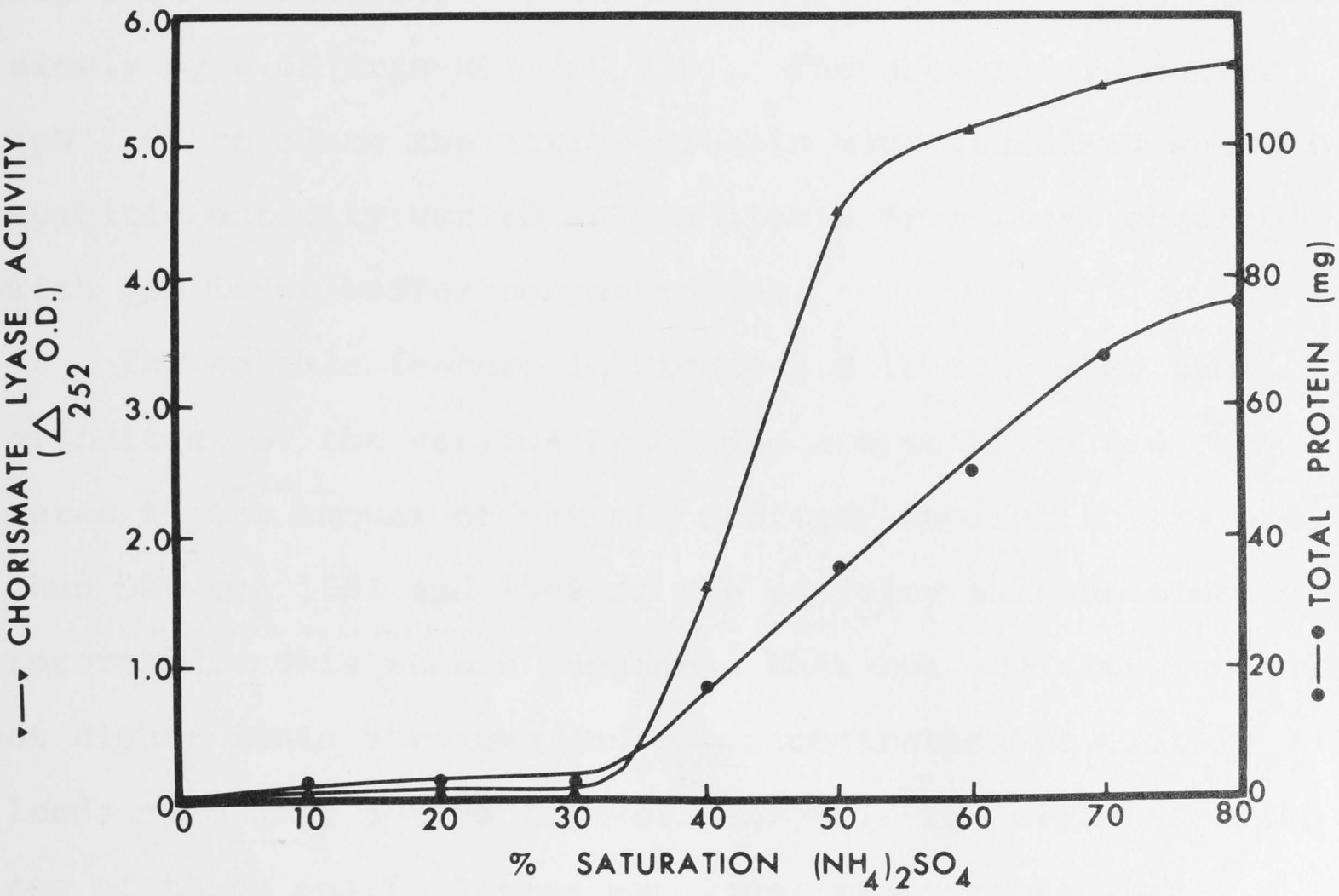


FIGURE 3.2 Fractionation of chorismate lyase activity with ammonium sulphate. Each sample contained 10ml of the protamine sulphate treated extract of strain AN58, to which differing amounts of solid ammonium sulphate were added, as described in MATERIALS AND METHODS.

The results of the elution of both protein and enzymic activity from the pad of DEAE-Sephadex (Figure 2.3) indicate that chorismate lyase binds very strongly to DEAE-Sephadex at low molar concentration of buffer and is eluted slowly by 0.1M Tris-HCl (pH 7.0). The use of 0.2M buffer (pH 7.0) to elute the active protein gave fractions whose specific activity varied only a little from those obtained with the lower buffer concentration.

The notable feature in Figure 3.3 is that when the activities of the various fractions are totalled and compared to the amount of enzymic activity applied to the pad, then between 150% and 250% of the activity is consistently recovered. This result suggested that the activity increases at higher ionic strength and/or concentration of protein leads to a less active form of enzyme. To determine which one of these possibilities was true, experiments were carried out to investigate (i) the effect of increasing buffer concentration, and therefore higher ionic strength, and (ii) the effect of concentrating the protein solution containing the enzyme. The results of Table II clearly shows the effect of using high and low buffer concentrations. The enzyme is more active at the higher molar concentration of 0.1M Tris-HCl buffer (pH 8.0) containing 10^{-3}M EDTA. However, concentration of the enzymic solution leads to a less rather than a more, active form of enzyme (Table III). The loss of activity is reversible for on dilution of the concentrated enzyme solution 80% of the original activity is regained immediately, while the remaining 20% is

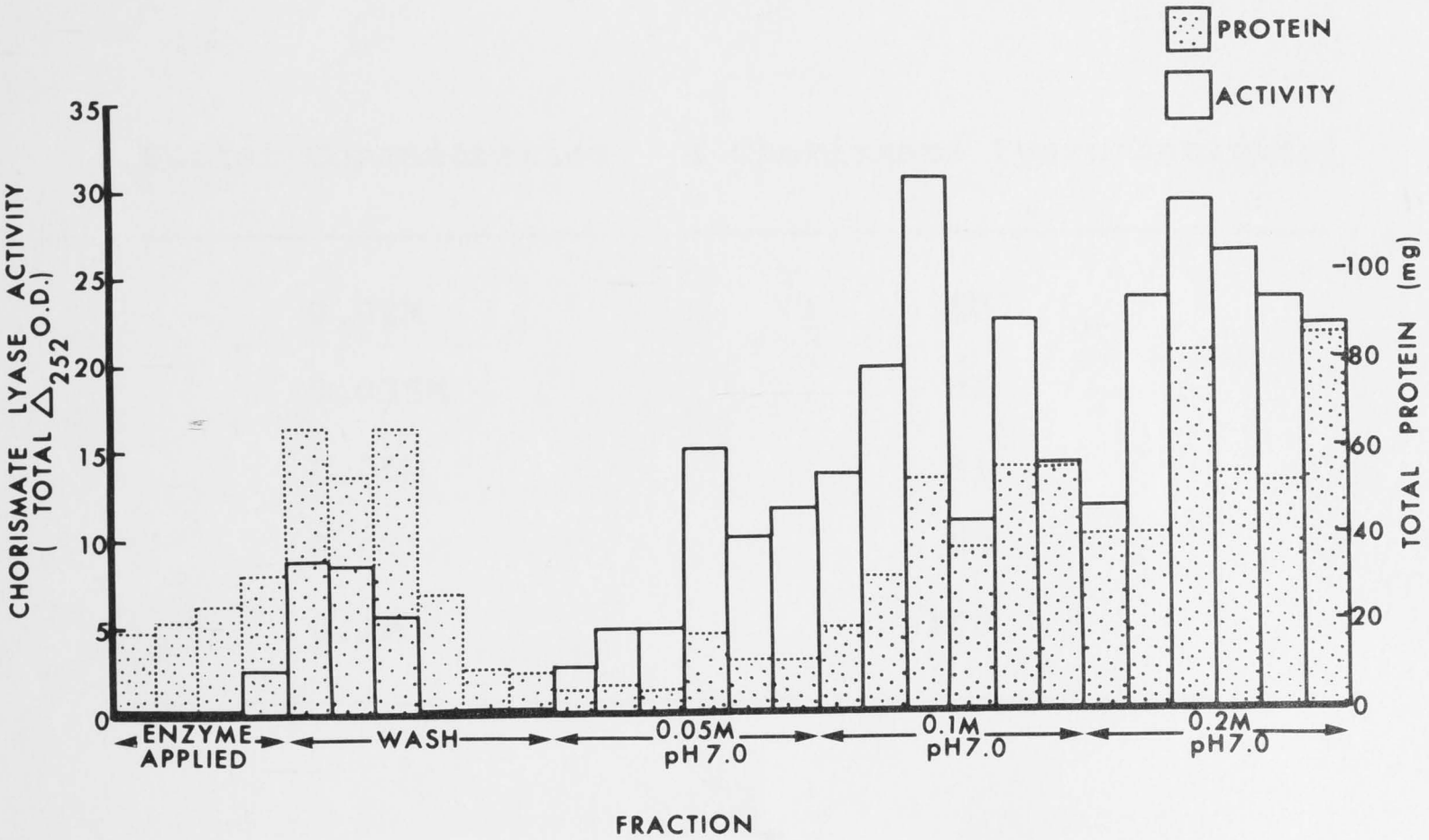


FIGURE 3.3 Batch elution of protein and chorismate lyase activity from a pad of DEAE-Sephadex. An ammonium sulphate fraction (150 ml) was applied to a pad of DEAE-Sephadex in 0.02M Tris-HCl (pH 8.0) containing 10^{-3}M EDTA. Chorismate lyase activity was eluted in 50 ml fractions, using Tris buffer of varying concentrations.

TABLE II. The Effect of Different Concentrations of Tris-Buffer on Chorismate Lyase Activity.

Buffer Concentration	% Chorismate Lyase Activity
0.02M	60
0.035M	82
0.05M	81
0.075M	83
0.1M	100

The purified preparation of chorismate lyase was used to determine the effects of different concentrations of Tris buffer.

Samples of enzyme were dialysed against the different buffer concentrations for 24 h before determining the activity as described in MATERIALS AND METHODS.

TABLE III. The Effect of Concentration of the Protein Solution.

Sample*	% Chorismate Lyase Activity
Before Concentration	100
After Concentration (10 fold)	65
Dilution of concentrated sample (1:10)	85
Dilution of concentrated sample (1:10) - assayed 24 hrs later	100

* The sample used was the eluate after batch treatment of the protein on DEAE-Sephadex.

recovered when the diluted solution is allowed to stand overnight at 4°C.

These experiments indicate that when the specific activity of the chorismate lyase preparation is low, enzymic activity is lost upon concentration, possibly due to the formation of complexes with inert protein. Another possible explanation is that these results could be due to a specific ion, namely Tris. Various other buffers were used to test this hypothesis (Table IV). However, the enzyme was most active in Tris buffer, and the effect of high and low buffer concentrations was the same regardless of the buffer used.

Since the loss and gain of activity was reproducible, it was decided to continue with the purification of the enzyme before undertaking further investigations of the effects of ionic strength and concentration on its activity.

(d) Column chromatography on DEAE-Sephadex.

Following the batch treatment of the enzyme on DEAE-Sephadex, fractionation using columns of DEAE and QAE-Sephadex was attempted as the next step in the purification. QAE-Sephadex was initially used, but on using a gradient of increasing molarity, the Sephadex compacted about 50%. Although both QAE and DEAE-Sephadexes behaved in this manner, DEAE-Sephadex did not compact as much as

TABLE IV. Effect of Different Buffer Concentrations on Chorismate Lyase Activity.

Buffer	ΔOD_{252} Chorismate Lyase Activity at two different buffer concentrations.	
	0.02M	0.1M
Tris-HCl-(pH 8.0)	0.12	0.21
N-Ethyl Morpholine-(pH 8.0)	0.08	0.14
TES - (pH 8.0)	0.13	0.16
HEPES - (pH 8.0)	0.09	0.15
Triethanolamine-HCl-(pH 8.0)	0.08	0.17

The purified preparation of chorismate lyase was used to determine the effects of the buffers on the activity. The enzyme was dialysed against the different buffers before determining the activity as described in MATERIALS AND METHODS.

QAE-Sephadex and was therefore preferred.

The protein was adsorbed to DEAE-Sephadex in 0.02M Tris-HCl buffer (pH 8.0) containing 10^{-3}M EDTA and the column was washed with a large volume of the same buffer to remove unadsorbed protein.

On increasing the buffer concentration to 0.1M the enzyme was slowly eluted. Therefore in order to obtain a sharp profile of chorismate lyase activity from DEAE-Sephadex, a linear gradient of Tris-buffer (0.02M - 0.2M) with a simultaneous pH gradient (pH 8.0 to pH 7.0) was used. A peak of activity coincident with a peak of protein was usually obtained. The active fractions were pooled and concentrated by ultrafiltration.

(e) Column chromatography by gel filtration.

(i) Agarose.

Following chromatography on DEAE-Sephadex, Agarose 0.5m was successfully used. In the early stages of development of the purification procedure multiple peaks of protein were sometimes demonstrated in the fractions after passing the protein solution through the Agarose column (Figure 3.4) This suggested that aggregation could be taking place and that active protein was being lost. Therefore active fractions from the Agarose column were combined, concentrated by ultrafiltration and passed alternately through Sephadex-G50 and Sephadex G-150 for comparison of recoveries.

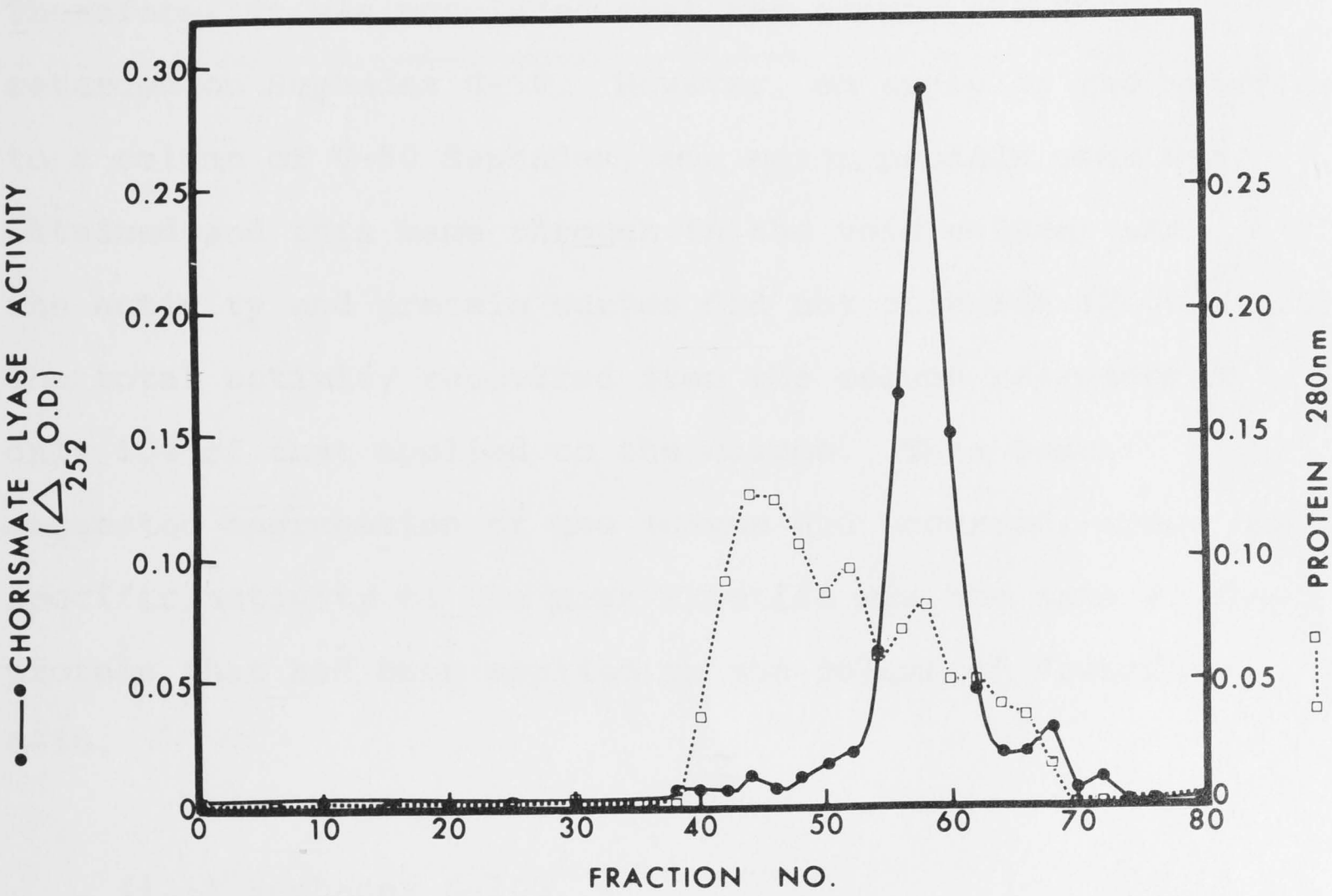


FIGURE 3.4 The elution of chorismate lyase activity after chromatography on Agarose 0.5m. The sample of protein (5 ml) was applied to a column of Agarose as described in the text. Fractions were collected in 3.0 ml volumes.

(ii) Sephadex G-50.

Preliminary experiments had shown that chorismate lyase was a small protein of molecular weight less than 30,000. Therefore, it was concluded that the enzyme should be retarded on Sephadex G-50. However, on applying the material to a column of G-50 Sephadex, one major protein peak was obtained and this came through in the void volume; also, the activity and protein curves did not coincide (Figure 3.5). The total activity recovered from the column represented only 10% of that applied to the column. This result suggested aggregation of the enzyme had occurred, since the specific activity of the peak fraction was the same as the protein that had been applied to the column of Sephadex-G-50.

(iii) Sephadex G-100.

The protein obtained after it had been passed through the column of Agarose in (i) was passed through a column of Sephadex G-100. One peak of protein coincident with the activity (Figure 3.6) was obtained. The total activity recovered was 95%.

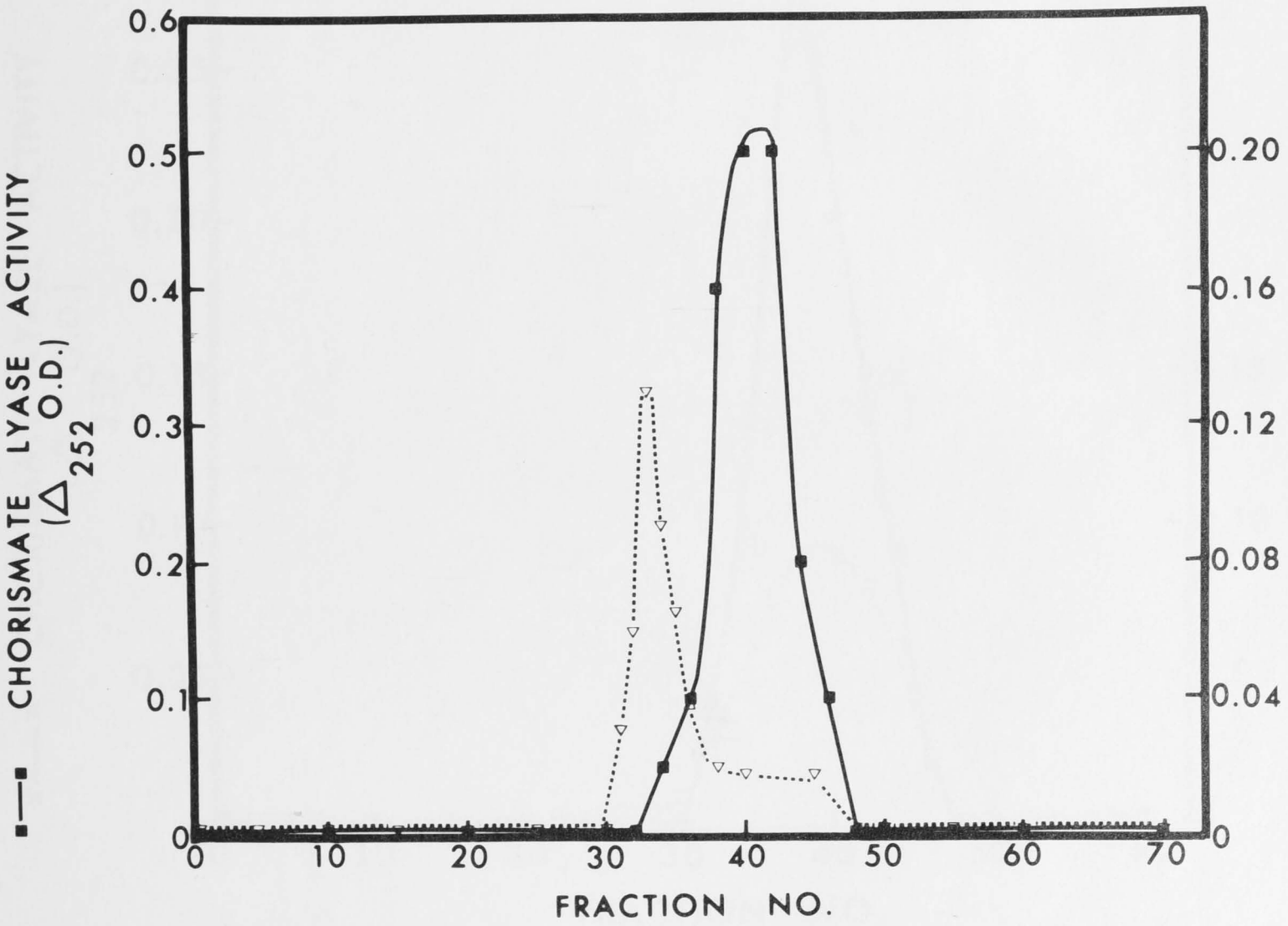


FIGURE 3.5 The elution of chorismate lyase activity after chromatography on Sephadex G-50. The sample of protein (3.3 ml) was applied to a column of Sephadex G-50, as described in the text. Fractions were collected in 3.0 ml volumes.

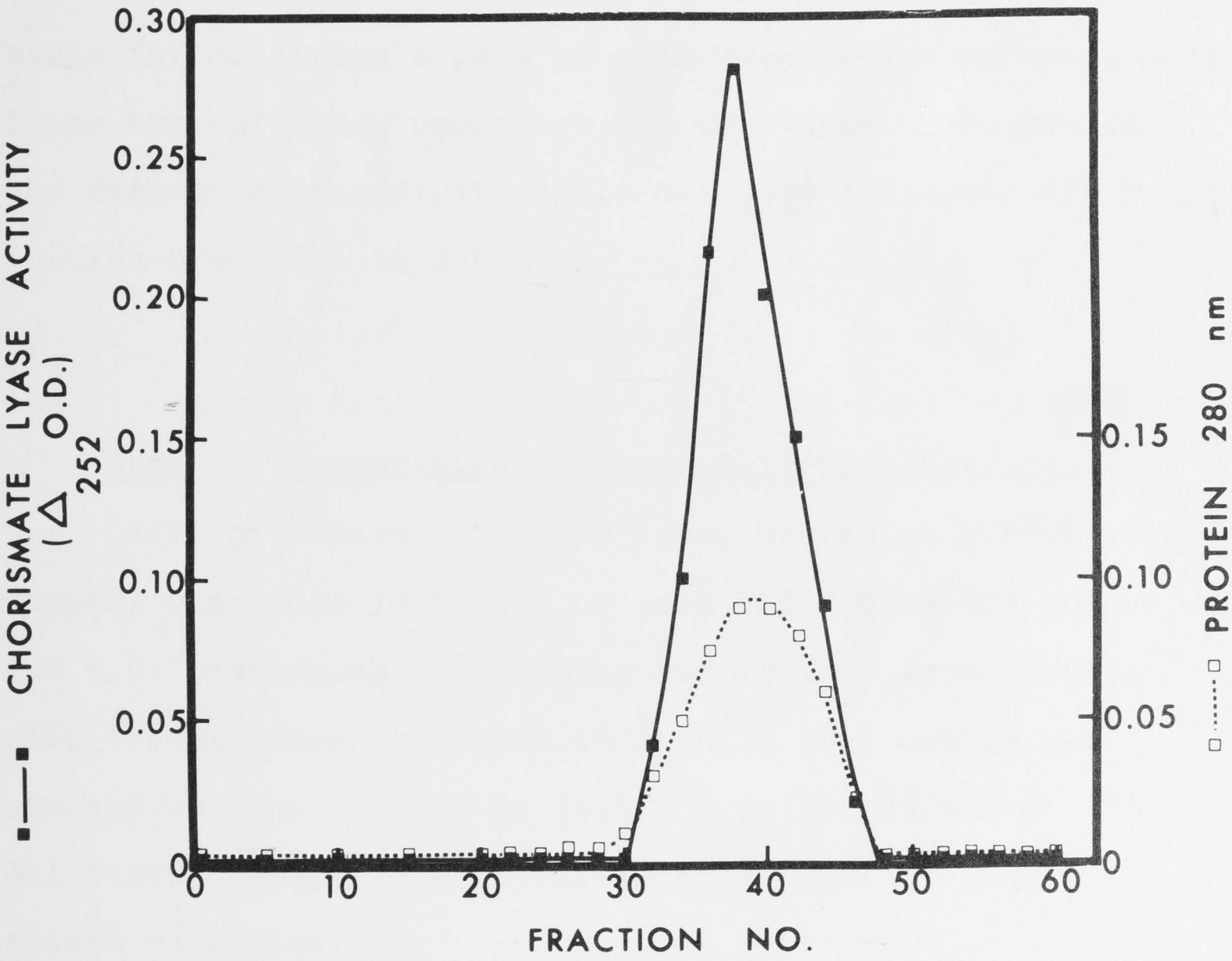


FIGURE 3.6 The elution of chorismate lyase activity after chromatography on Sephadex G-100. The sample of protein (5.0 ml) was applied to a column of Sephadex G-100, as described in the text. Fractions were collected in 3.0 ml volumes.

Purification Procedure for Chorismate Lyase.

Using the preliminary experiments described above as a basis for obtaining a pure protein preparation of chorismate lyase the following procedure was developed. The yields and degrees of purification are outlined following the Purification Procedure in Table V.

Step 1. Preparation of Crude Cell-Free Extracts.

Cells of strain AN58 (170-180g, stored at -20°C) were thawed, suspended in 3 vol. of cold 0.1 M Tris-HCl buffer (pH 8.0) containing 10^{-3}M EDTA, and smashed in a Sorvall Ribi Fractionator at 20,000 lb/inch^2. Cell debris was removed by centrifuging at 17,500 x g, for 30 min at 4°C. All further steps were performed at 0° to 4°C, unless stated otherwise.

Step 2. Removal of Nucleic Acid.

To determine the amount of protamine sulphate required to remove the nucleic acid, a pilot scale experiment was carried out. A 2% solution of protamine sulphate in 0.1M Tris-HCl buffer (pH 8.0) containing 10^{-3}M EDTA was added dropwise with stirring to the solution of cell-free extract. The mixture was stirred for a further 30 min after the final addition of protamine sulphate and then centrifuged at 13,700 x g for 20 min.

Step 3. Ammonium Sulphate Fractionation.

Finely ground ammonium sulphate (31.4g/100 ml) was added to the supernatant from the previous step over a period of 3 h. After the final addition of ammonium sulphate, the mixture was stirred for a further 30 min and then centrifuged at 13,700 x g for 15 min. The precipitate was redissolved in 150 ml 0.02M Tris-HCl buffer (pH 8.0) containing 10^{-3}M EDTA and the protein solution was dialysed overnight against 4 l of the same buffer at 4^{o}C.

Step 4. Batch Elution.

The dialysed solution from step 3 was applied to a Buchner funnel, containing a pad (12 x 5 cm) of DEAE-Sephadex that had been previously equilibrated with 0.02M Tris-HCl buffer (pH 8.0) containing 10^{-3} M EDTA. After the protein had been applied, a further 700 ml of the same buffer was used to wash the pad in order to remove the unadsorbed protein. The enzyme was eluted by allowing 1.2 l of 0.1 M Tris-HCl buffer (pH 7.0) containing 10^{-3} M EDTA to slowly seep through the pad under reduced pressure. Care was taken to avoid frothing of the eluate, which could cause denaturation of the enzyme. The eluate was concentrated to 40-50 ml using a Diaflo ultrafiltration cell with a UM-2 membrane.

Step 5. DEAE-Sephadex Chromatography.

The concentrated solution from step 4 was dialysed overnight against 4 l of 0.02M Tris-HCl buffer (pH 8.0) containing 10^{-3}M EDTA, and then applied to a column (2 x 40 cm) of DEAE-Sephadex equilibrated with the same buffer. The column was then washed with sufficient buffer (usually 500 ml) to remove all unadsorbed protein. Elution of the enzyme was carried out with a linear gradient of Tris buffer (0.02M - 0.2M), with a simultaneous pH gradient (pH 8.0 to pH 7.0), in a total volume of 500 ml (Figure 3.7). The active fractions were pooled and concentrated by Diaflo ultrafiltration to a volume of approximately 5 ml.

Step 6. Gel Filtration on Agarose.

The concentrated solution from step 5 was dialysed against 2 l of 0.1 M Tris-HCl buffer (pH 8.0) containing 10^{-3}M EDTA, and applied to a column (1 x 136 cm) of Agarose equilibrated with the same buffer. The enzyme was eluted with the same buffer and the active fractions were pooled and concentrated by Diaflo ultrafiltration, to a volume of approximately 5 ml (Figure 3.8).

Step 7. Gel Filtration on Sephadex G-100.

When more than one peak of protein was obtained from the Agarose column, it was necessary to apply the protein to Sephadex G-100. This was done to ensure that there was

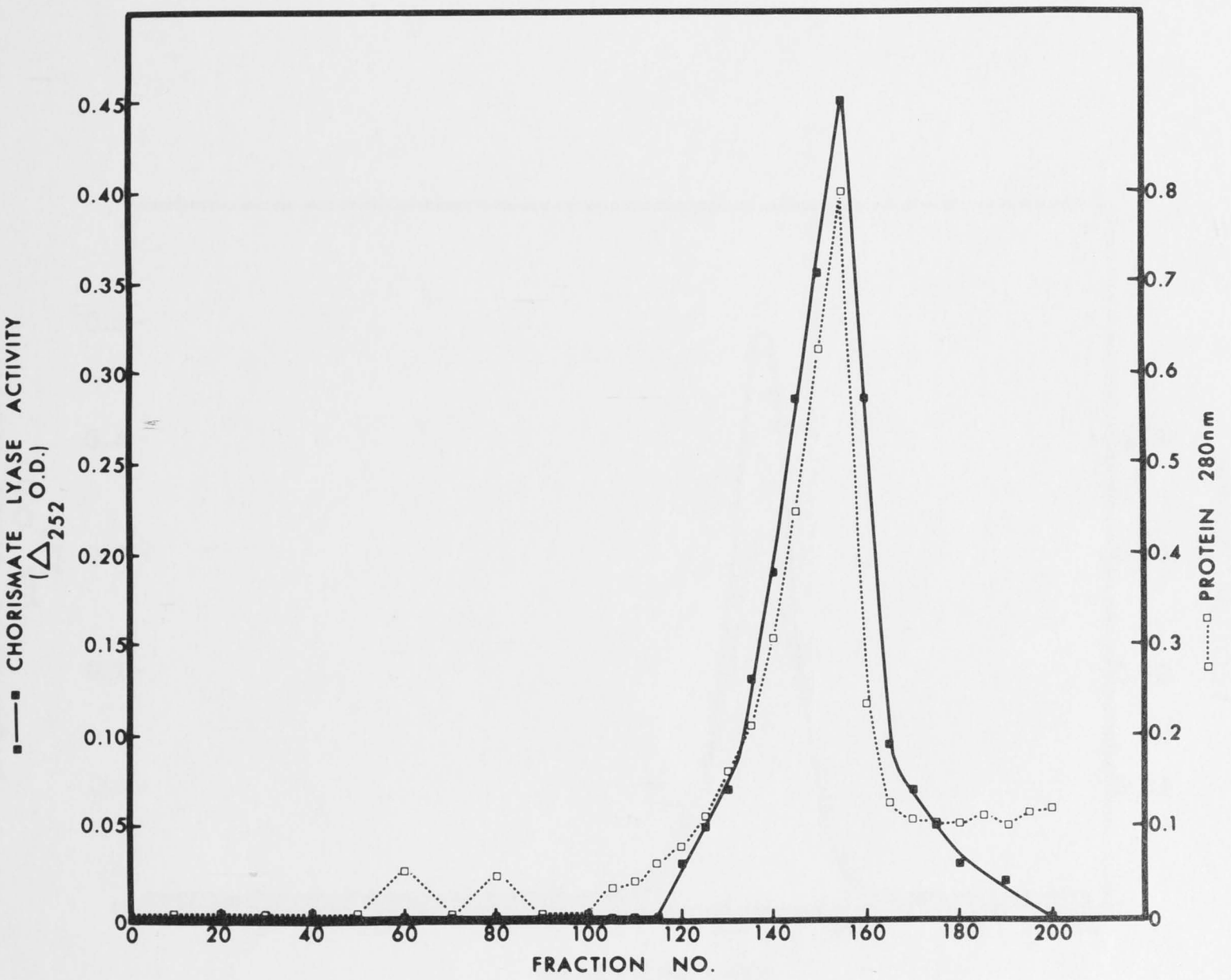


FIGURE 3.7 The elution of chorismate lyase activity after chromatography on DEAE-Sephadex A-50. Enzymic activity was eluted using a linear gradient of Tris buffer (0.02M - 0.2M) with a simultaneous pH gradient (pH 8.0 to pH 7.0).

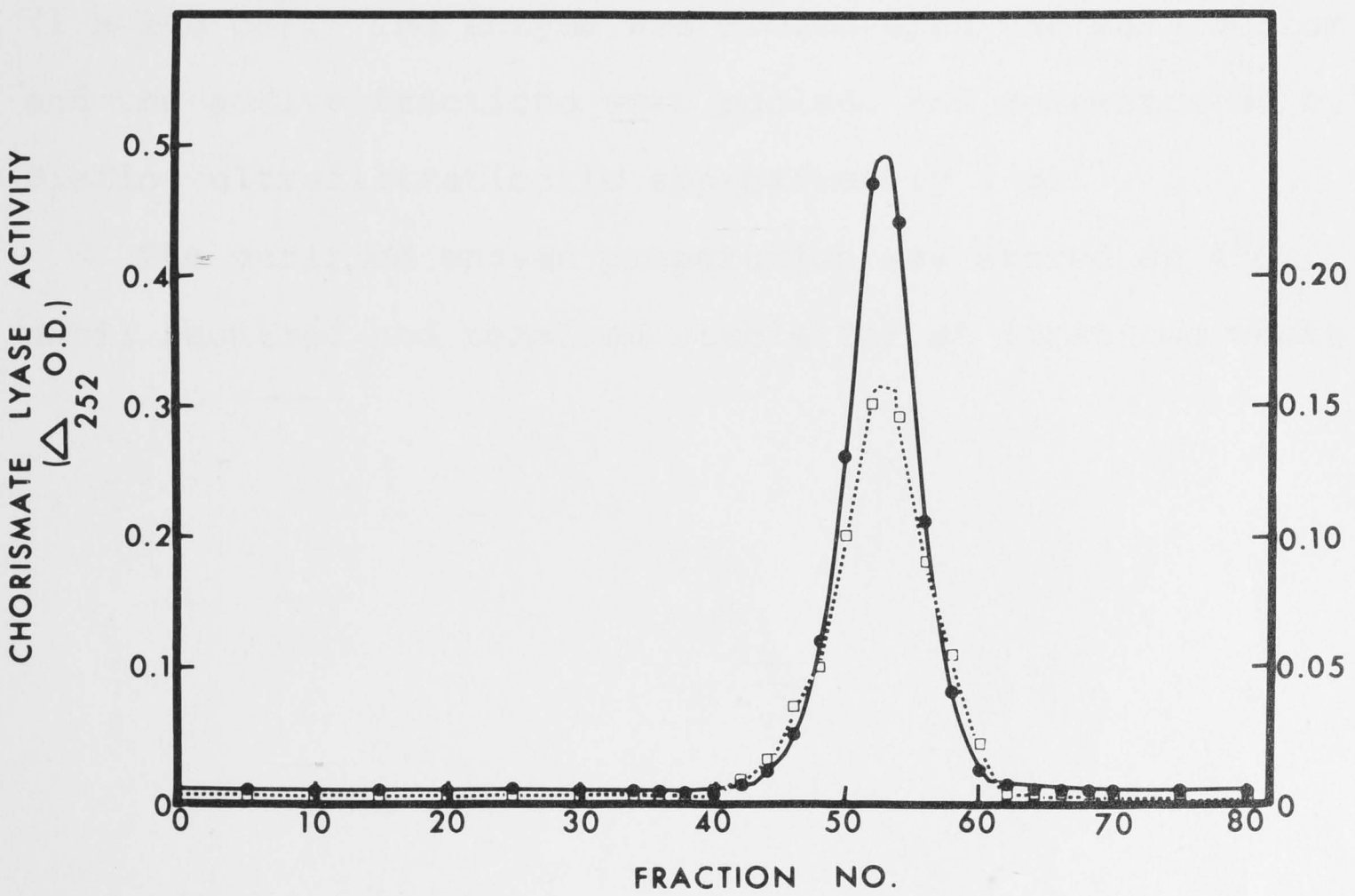


FIGURE 3.8 Elution of chorismate lyase activity after chromatography on a column of Agarose.

□ ---□, protein; ● —●, chorismate lyase activity

only one peak of protein, which was coincident with the activity.

Under these circumstances the protein obtained from step 6 was dialysed against 2 l of 0.1M Tris-HCl buffer (pH 8.0) containing 10^{-3}M EDTA and applied to a column (1 x 100 cm). The enzyme was eluted with the same buffer and the active fractions were pooled, and concentrated by Diaflo ultrafiltration to approximately 5 ml.

The purified enzyme preparation was stored at 4^{o}C until required and remained stable for at least two weeks.

TABLE V. Purification of Chorismate Lyase From E. coli K-12.-

Summary of yields and degrees of purification.

Procedure	Vol. (ml)	Activity (units/ml)	Total Units	Total Protein (mg)	Specific Activity (units / mg)	Purification.	Yield (%)
Crude extract	550	3.5	1,925	16,775	0.11	1	100
Supernatant after precipitation of nucleic acid.	800	2.6	2,080	11,440	0.18	2	108
0-0.5 Sat. $(NH_4)_2SO_4$ ppt. assayed after dialysis.	150	4.8	720	3,075	0.23	2	37
Eluate from Batch treatment on DEAE-Sephadex							
1. before concn.	1200	1.14	1,368	398	3.43	30	71
2. after concn.	40	6.8	272	398	0.68	6	14
DEAE-Sephadex column	56	0.91	51	16	3.3	28	3
Agarose column	4	6.33	25	7	3.6	33	1

DISCUSSION.

The development of the purification procedure for chorismate lyase was very time consuming, especially in the early stages, because of the complex behaviour of the enzyme. Further, the low concentration of enzymic activity in crude extracts made the task of obtaining a pure preparation a difficult one. Many different assay procedures were tried in the early stages of the purification, but failed due to (i) the presence of other contaminating enzymes or (ii) the mixture of products obtained from the chemical decomposition of the substrate, chorismate. These technical difficulties were avoided by the use of a spectrophotometric assay for 4-hydroxybenzoate, even though this was a time consuming method.

The apparent inexplicability of a large number of the initial observations was almost wholly due to aggregation or association of chorismate lyase with other inert protein. These effects complicated the interpretation of results very markedly during the purification procedure, and made it very difficult to place much confidence in the results obtained until the system was more clearly understood. From the results showing the degree of purification and the yield of the pure protein preparation (Table V), the values for the specific activities do not increase in magnitude

step by step, as would be expected. This is particularly noticeable before and after concentration of the eluate from the batch treatment of protein on DEAE-Sephadex, where the specific activity decreases and then increases again after concentration. This is explained by the previous observation of the effect of concentration of the enzyme solution (Table III).

The enzyme preparation usually showed a constant specific activity after the Agarose column. If this was not so, the active fractions from the Agarose column were combined, concentrated and passed through a column of Sephadex G-100 in order to obtain such a preparation. The enzyme preparation was stored at $4^{o}C$ and maintained the same specific activity for a period of at least two weeks.

Due to the possibility that aggregation of chorismate lyase could cause grossly inaccurate values in degrees and yields of purification (Table V) the amount of enzyme in the cell has not been given.

SECTION 3.2. PROPERTIES OF CHORISMATE LYASE.

INTRODUCTION.

One of the aims of the present work was to determine whether the enzyme had a high or low turnover number. Coupled with this, the general properties of chorismate lyase were also investigated with a view to further understanding the functional characteristics of the enzyme.

In the previous section, details of the purification procedure are given and as a continuation to this, the homogeneity of the protein preparation is established. The behaviour of chorismate lyase on polyacrylamide gels, in 8M urea, and in sodium dodecyl sulphate is described. The homogeneity of the protein preparation is established using the criteria of ultracentrifugation and polyacrylamide gel electrophoresis. The molecular weight of the protein preparation is determined by gel filtration and sedimentation equilibrium experiments, together with amino acid analysis and peptide maps.

Lastly, some of the kinetic parameters are determined and an attempt to draw some conclusions as to the possible mechanism of the reaction is made from these results.

MATERIALS AND METHODS.

Chemicals.

Chemicals generally were of the highest purity obtainable commercially and were not further purified.

Iodoacetamide was purified by recrystallisation from ethyl acetate-petroleum ether and stored in the dark until used. Barium prephenate was prepared by Dr. R. Ghambeer, and chorismate was prepared by the method of Gibson (1968).

Determination of Enzyme Activity.

The conversion of chorismate to 4-hydroxybenzoate and pyruvate was measured in two ways:-

(i) Initial rates for the conversion were measured by stopping the reaction at fixed time periods, followed by spectrophotometric estimation of 4-hydroxybenzoate. The reaction mixture contained 1.66 mM chorismate, 0.4 mM EDTA, chorismate lyase (~200 µg protein) and 40 mM Tris-HCl buffer (pH 8.0), in a final volume of 1.0 ml. The blank consisted of all the reactants except the enzyme. After incubation at 37°C for a specific time period, the reaction was terminated by the addition of 0.5 ml of

0.2M sodium acetate buffer (pH 4.0). 4-Hydroxybenzoate was extracted into ether, (5 ml), which was dried over anhydrous sodium sulphate before determination of absorbency on a Model 15 Cary Spectrophotometer from 360-240 nm. The amount of 4-hydroxybenzoate formed was calculated using a molar extinction value of 16,400 in ether, at 252 nm.

(ii) Initial rates for the reaction were measured continuously in a Cary 14 Recording Spectrophotometer in the presence of the coupled enzyme system of lactate dehydrogenase/NADH. The amount of pyruvate formed was measured by the disappearance of NADH at 340 nm. The reaction mixture for the inhibition studies consisted of 1 to 10 mM chorismate, 0.4 mM EDTA, 50 µl chorismate lyase (~200 µg protein), 0.1 mM NADH, 5 µg lactate dehydrogenase, and 250 mM N-Ethyl Morpholine-HCl buffer (ph 8.0), in a final volume of 1.0 ml. The blank consisted of all the reactants except the enzyme.

Where inhibitors and various analogues were added, the volumes and concentrations of other components remained unaltered. The reaction mixtures were preheated at 37^{o}C in a cuvette in the heated compartment of the Cary Model 14 spectrophotometer and the reaction was commenced by the addition of enzyme solution. The amount of NAD formed was calculated using a molar extinction coefficient of 6,220 (Dawson et al. 1969).

Methods of Analysis of Kinetic Data.

The calculations of the Michaelis-Menten constant Km, the maximal velocity and inhibitor constant, Ki, were done using two programs provided and calculated by Dr. J.F. Morrison. The programs were based on the method of minimisation of the sum of the squares of the distances of the points from the line.

Equation (1) was $v = \frac{V}{1 + \frac{Km}{s}}$

and Equation (2) was $v = \frac{V}{1 + \frac{Km}{s}\left(1 + \frac{i}{Ki}\right)}$

Polyacrylamide Gel Electrophoresis.

Polyacrylamide disc gel electrophoresis was carried out using essentially the same method as that described by Davis (1964), except that the stacking and sample gel were eliminated. The sample in 0.1M Tris-HCl buffer (pH 8.0) containing 10^{-3} M EDTA and 40% sucrose was applied directly to the top of the resolving gel. The two buffer systems used were Tris-glycine in the range from pH 9 to pH 10 (Davis, 1964) and the Triethanolamine (TEA) - TES-chloride system in the range pH range 7-8 (Orr, 1969). Bromophenol blue was added to each gel and runs were made at 4^{o}C at 2.5 mA/tube. Protein was detected by staining the gel with Amido Black in acetic acid (Davis, 1964).

Gel Electrophoresis using Sodium Dodecyl Sulphate.

Protein was denatured by the method of Weber and Osborn (1969), which uses sodium dodecyl sulphate. Protein solutions were dialysed overnight at room temperature against 0.01M sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulphate. The samples (∿100 µg) were then electrophoresed in either 7.5% or 10% gels at room temperature with a constant current of 4 mA/tube. The gels contained 0.2% sodium dodecyl sulphate. Protein was stained as described above.

Electrophoresis in 8M urea.

Electrophoresis of proteins in polyacrylamide gels containing 8M urea was carried out using the Tris-glycine buffer pH 9.0 - 10.0 of Davis (1964). 8M urea was added to 10% gels before polymerisation and the samples were also prepared in 8M urea.

Ultracentrifugation.

Sedimentation velocity runs were used to establish the purity of the enzyme preparation. These were carried out in the Model E Analytical Ultracentrifuge at 60,000 revolutions per min in aluminium-filled capillary-type synthetic boundary cells at a temperature of approximately 21^{o}C.

To determine the approximate molecular weight, $M_w^{app.}$, equilibrium ultracentrifugation was used. The conditions were calculated according to the scheme of Howlett and Nichol (in press). The plates were measured and the results computed using a program of G. Howlett which was based on the method of Richards, Teller, and Schachman (1968). The ultracentrifugation work to determine the molecular weight of chorismate lyase was performed by Dr.A.B. Roy of the Department of Physical Biochemistry, Australian National University.

Gel Filtration and Molecular Weight Determination.

The molecular weight of chorismate lyase was determined by gel filtration according to the method of Andrews (1964, 1965) using a column of Sephadex G-100 (1 x 100 cm) equilibrated with 0.1M Tris-HCl (pH 8.0) buffer containing 10^{-3}M EDTA. Samples of the standard protein solution (10 mg/ml) were applied to the column in 1 ml volumes of the same buffer. Fractions were collected and the exact volume in each fraction was measured by weighing each tube before and after the samples were run.

Peptide Mapping.

The purified enzyme (2 mg) was hydrolysed by trypsin (1% w/w) in aqueous ammonium bicarbonate solution (0.5%) pH 8.0 at 37°C for 2 h after which time a further aliquot of trypsin (1% w/w)was added and hydrolysis was continued for a further 2 h; to destroy any chymotryptic activity, trypsin was allowed to stand in ice in 0.01M hydrochloric acid for ½ h before use. The ammonium bicarbonate was removed by lyophilisation, and the hydrolysate was dissolved in a small volume of water (approximately 20 µl). The soluble peptides were applied to a sheet of Whatman 3MM paper and subjected to high voltage electrophoresis in pyridine-acetic acid buffer at pH 4.7, in tanks designed after Michl (1958), using varsol (mineral turpentine) as heat exchanger. The buffer system contained 25 ml pyridine and 25 ml glacial acetic acid in 1 ℓ (Schwartz, 1963).

After the electrophoretograms had been dried at room temperature, the strips of paper containing the separated peptides were sewn onto fresh sheets of paper (Milstein and Sanger, 1961) and then chromatographed in the second dimension using n-butanol: acetic acid: water (4:1:5 by vol., upper phase only; Katz, Dreyer and Anfinsen, 1959) or pyridine: isoamyl alcohol: water (7:7:6 by vol.; Wittman and Braunitzer, 1959). During the preparation of the peptide maps for chorsimate lyase, β-lactoglobulin was run as a control.

Each chromatogram was sprayed with ninhydrin reagent (0.1% w/v ninhydrin in acetone, containing 0.2% v/v glacial acetic acid and 0.2% v/v pyridine), and stored in the dark for 24 h to allow colour development. The chromatograms were photographed and the ninhydrin-positive areas were marked with pencil before decolourisation with chlorine. Larger peptides and in particular the 'core' material which does not stain well with ninhydrin, were detected after chlorination by starch-iodide (Rydon and Smith, 1952).

Preparation of Dansyl Peptide Maps.

Twice recrystallised trypsin (1%) was treated with $\frac{M}{16}$ hydrochloric acid for 24 h at 37°C (Redfield and Anfinsen, 1956), and then dialysed against cold water for 24 h before being lyophilised. Tryptic digestion was carried out in 0.1M sodium bicarbonate (pH 8.0), using 5% (w/w) of trypsin for 2 h at 37°C. The tryptic digestion mixture was then added dropwise to an equal volume of a solution of 'dansyl' chloride (twenty-fold excess) in 0.1M sodium bicarbonate and acetone (1:1 by vol.; Atherton and Thomson, 1969). The resulting mixture was left to stand at room temperature overnight before applying to a column of Dowex 50 x 8 (1 x 2 cm) previously equilibrated with 0.01M acetic acid. The column was washed with 600 ml of 0.01M acetic acid before eluting the peptides with 1M

ammonia in 25% aqueous acetone (v/v). The eluant, containing fluorescent material, was concentrated by rotary evaporation. The sample containing the peptides was dissolved in a minimum volume of 90% aqueous tetrahydrofuran (v/v) before applying to a thin layer silica gel plate (20 x 20 cm; 2.5 mm thick) and developing in a two dimensional chromatographic system. Solvent 1 (first dimension) consisted of methyl acetate:propan-2-ol:aqueous NH_3 (9:7:4 by vol.; Seiler and Wiechmann, 1964), and solvent 2 (second dimension) consisted of chloroform: 95% ethanol: acetic acid (38:4:3 by vol.; Deyl and Rosmus 1965).

Amino Acid Analysis.

The protein samples were hydrolysed in sealed evacuated tubes in 6M HCl at 110°C for 22 h (Crestfield, Moore and Stein, 1963). The hydrolysates were analysed essentially by the method described by Spackman, Stein and Moore (1958) using a Beckman Model 120B amino acid analyser. Half cysteine was determined as cysteic acid after performic acid oxidation of the protein (Hirs, 1956). Tryptophan was not estimated.

Identification of the Reaction Products.

The conversion of chorismate to 4-hydroxybenzoate was established by Gibson and Gibson (1962). The other product which was derived mechanistically from this reaction, was established by (i) chromatography and (ii) a coupled enzyme system using NADH and lactate dehydrogenase. The reaction mixture consisted of 4 mM chorismate, 90 mM Tris-HCl buffer (pH 8.0), 0.9 mM EDTA and 200 µg chorismate lyase in 1.0 ml. The control consisted of all the reactants except the enzyme. The reaction was incubated at 37°C for 4 h and terminated in the usual way by the addition of 0.5 ml 0.2M sodium acetate buffer (pH 4.0). The 2,4 dinitrophenylhydrazone derivative of the product formed was prepared by the method of Neuberg, Grauer and Pisha (1952) and chromatographed and developed on thin layer plates of silica gel in benzene: tetrahydrofuran: acetic acid (60:36:4 by vol.; Byrne, 1965).

Stoichiometry of the Reaction.

Estimation of the amount of 4-hydroxybenzoate formed was determined by the normal spectrophotometric method used previously, while pyruvate was estimated spectrophotometrically at 340 nm using the coupled enzyme system, lactate dehydrogenase and NADH. Samples were taken at

various time intervals in order to determine when equilibrium was established. When estimating 4-hydroxybenzoate, the reaction was terminated by adding the sample to 0.5 ml 0.2M sodium acetate buffer (pH 4.0). The aliquot (0.1 ml) for the estimation of pyruvate was diluted 10 fold which effectively terminated the reaction.

Protein Determination.

The protein concentration in samples of purified enzyme was obtained spectrophotometrically by determining the absorption coefficient at 280 nm of a 0.1% solution (w/v). The absorption coefficient was calculated to be 1.40.

RESULTS.

Homogeneity of the Purified Enzyme.

(a) Ultracentrifugation.

Ultracentrifugation of the enzyme gave a single symmetrical peak which suggested that it was a homogeneous preparation (Figure 3.9). An S_{20} value of 2.0 was obtained using buffer concentrations of 0.1 and 0.01M. Although an $S^{o}_{20,w}$ value is not available, it would not differ from the S_{20} value by more than 5%. As the $S^{o}_{20,w}$ for lysozyme (molecular weight 14,000) is approximately 1.87, the estimated S_{20} value for chorismate lyase would suggest a molecular weight of about 15,000.

(b) Polyacrylamide Gel Electrophoresis.

When the gels were run in two different buffer systems, namely in the system described by Davis (pH 9-10) (1964) and the system described by Orr (pH 7-8) (1969), multiple bands of protein were always seen (Figure 3.10). These consisted of three major bands together with a number of minor bands. However, on treating the protein with sodium dodecyl sulphate, as described by Weber and Osborn (1969), one band of protein was obtained (Figure 3.10). Since sodium dodecyl sulphate reacts with

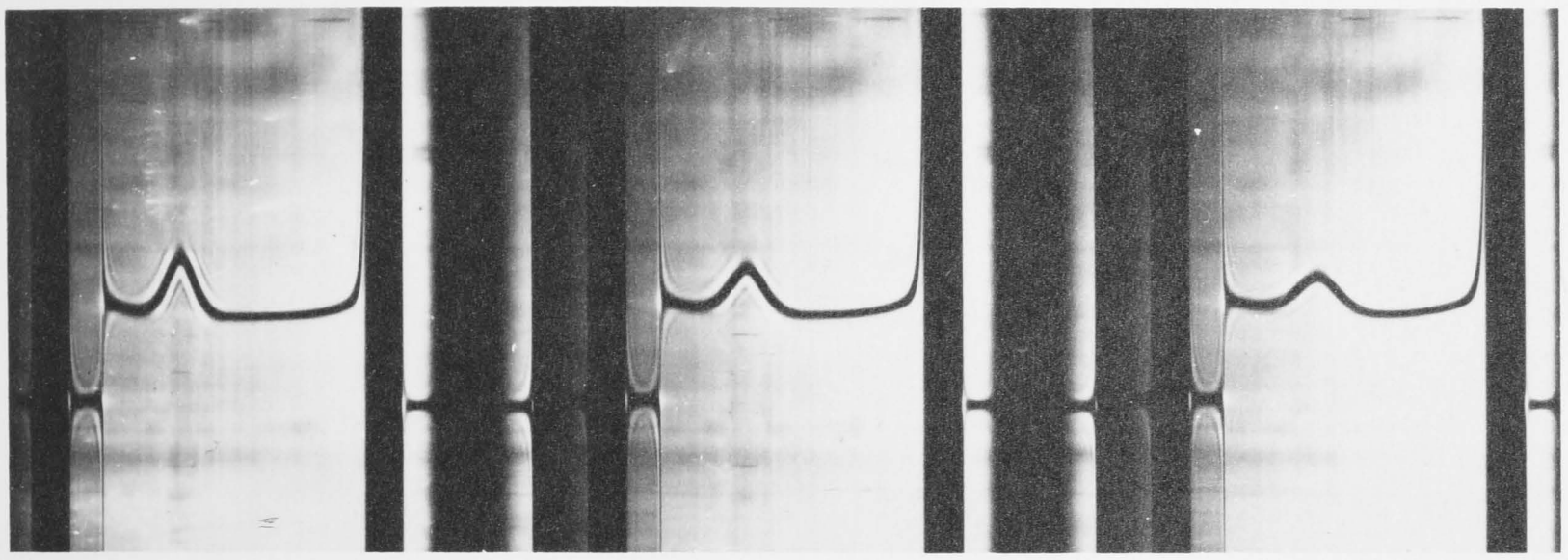


FIGURE 3.9 The sedimentation pattern of purified chorismate lyase protein. The enzyme concentration was 2.7 mg per ml in a medium of 0.05M NaCl and 0.05M HCl buffered at pH 8.0 with Tris. Photographs were taken at 8 min intervals and an S_{20} value of 2.0 suggested a molecular weight of ∿15,000.

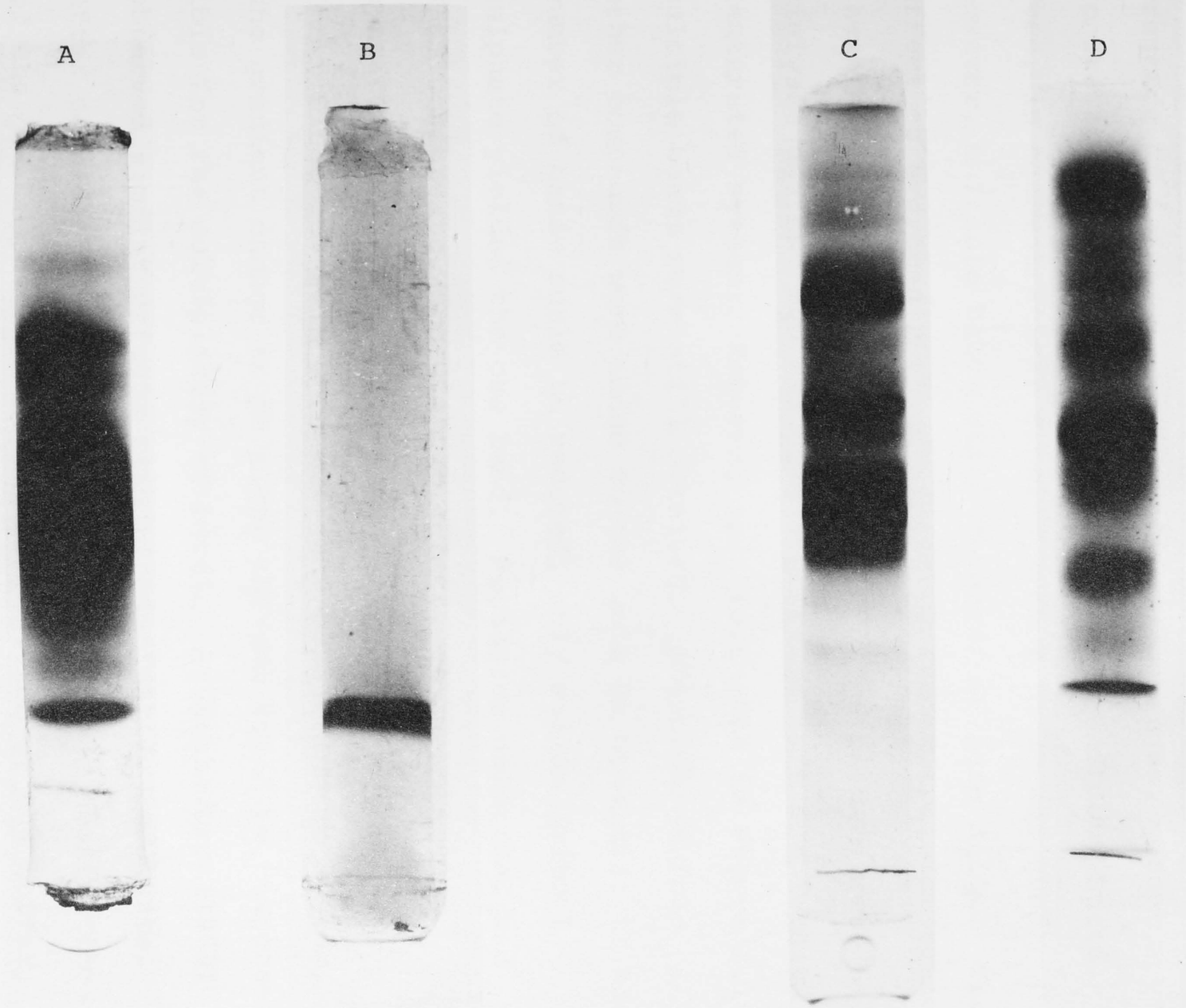
FIGURE 3.10 Polyacrylamide gel electrophoresis of ~100 µg samples of chorismate lyase :

A. run in Tris-glycine (pH 9.0 - 10.0)

B. run in Tris-glycine in the presence of 0.1% sodium dodecyl sulphate

C. run in TES-TEA (pH 7.0 - 8.0)

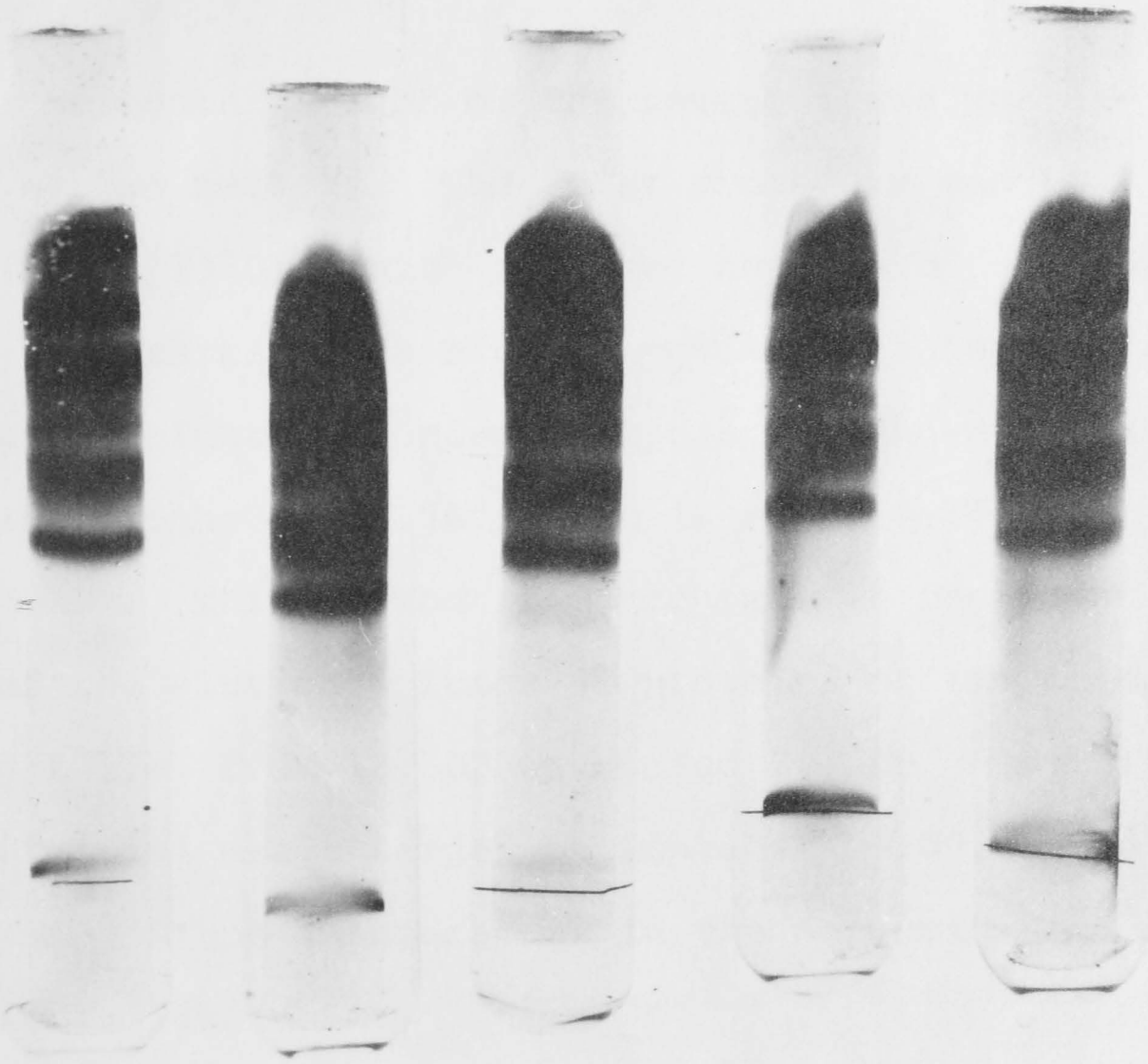
D. run in a completely deuterated system using Tris-glycine buffer.


A
B
C
D

protein and overcomes charge effects, movement of protein in gels depends only on its molecular weight. The behaviour of the protein on normal gels could suggest the presence of isoenzymes with different charges. Other denaturating agents were tested and their effects noted. Urea, in concentrations ranging from 2 to 8M was used; however, multiple bands still appeared (Figure 3.10a. The effect of hydrogen bonding as a possible explanation for these observations was investigated. The protein was dialysed against D_2O and electrophoresed in a completely deuterated system. However, as illustrated in Figure 3.10, multiple bands were still obtained. Although various other compounds were added to the gels to determine if the number of bands could be reduced, only sodium dodecyl sulphate yielded the one band. Potassium chloride, and 4-aminobenzoate were without effect, while potassium cyanate yielded a reduced number of bands.

Since the gels were run in a discontinuous system, the gradient change in pH along the gel could be responsible for the multiplicity of bands. In fact, Orr (1969) observed similar effects with ribonucleotide reductase. After considerable effort varying both the concentrations of the gel components and the composition of the buffer, he was able to obtain a single band of protein.

It was the effect of sodium dodecyl sulphate in gels that indicated the preparation of chorismate lyase was purified to homogeneity.



A B C D E

FIGURE 3.10a Polyacrylamide gel electrophoresis using different concentrations of urea. Samples of chorismate lyase were treated with urea and then electrophoresed in gels containing different concentrations of urea :

A. 2 M; B. 4 M; C. 6 M; D. 8 M;

E. no urea.

Molecular Weight Determinations.

The molecular weight of chorismate lyase was determined by two methods. The first procedure was that of Andrews (1964, 1965), which involved the use of standard proteins to determine the elution volume with respect to molecular weight. Chorismate lyase was shown to have a molecular weight of 15,800 which is relatively small (Figure 3.11). This method is inaccurate in the determination of the elution volumes of proteins of low molecular weight i.e. from 12,500 to around 20,000. In this region, proteins are retarded on Sephadex G-100, but give broad peaks. Thus inaccuracies in the determination of the elution volume arise.

The molecular weight was also determined by equilibrium ultracentrifugation, which gives an approximate value by measuring the molecular weight at various concentrations of protein. Hence, a range of molecular weights is obtained. For a pure non associating-dissociating system, only one value is obtained. However, in the present case, a range of molecular weights was obtained ranging from a value of approximately 13,000 at the meniscus to 39,000 at the base of the cell (Figure 3.12). The conclusions that may be drawn from this are (i) that the protein is inhomogeneous or (ii) the protein is undergoing association-dissociation under these conditions. This

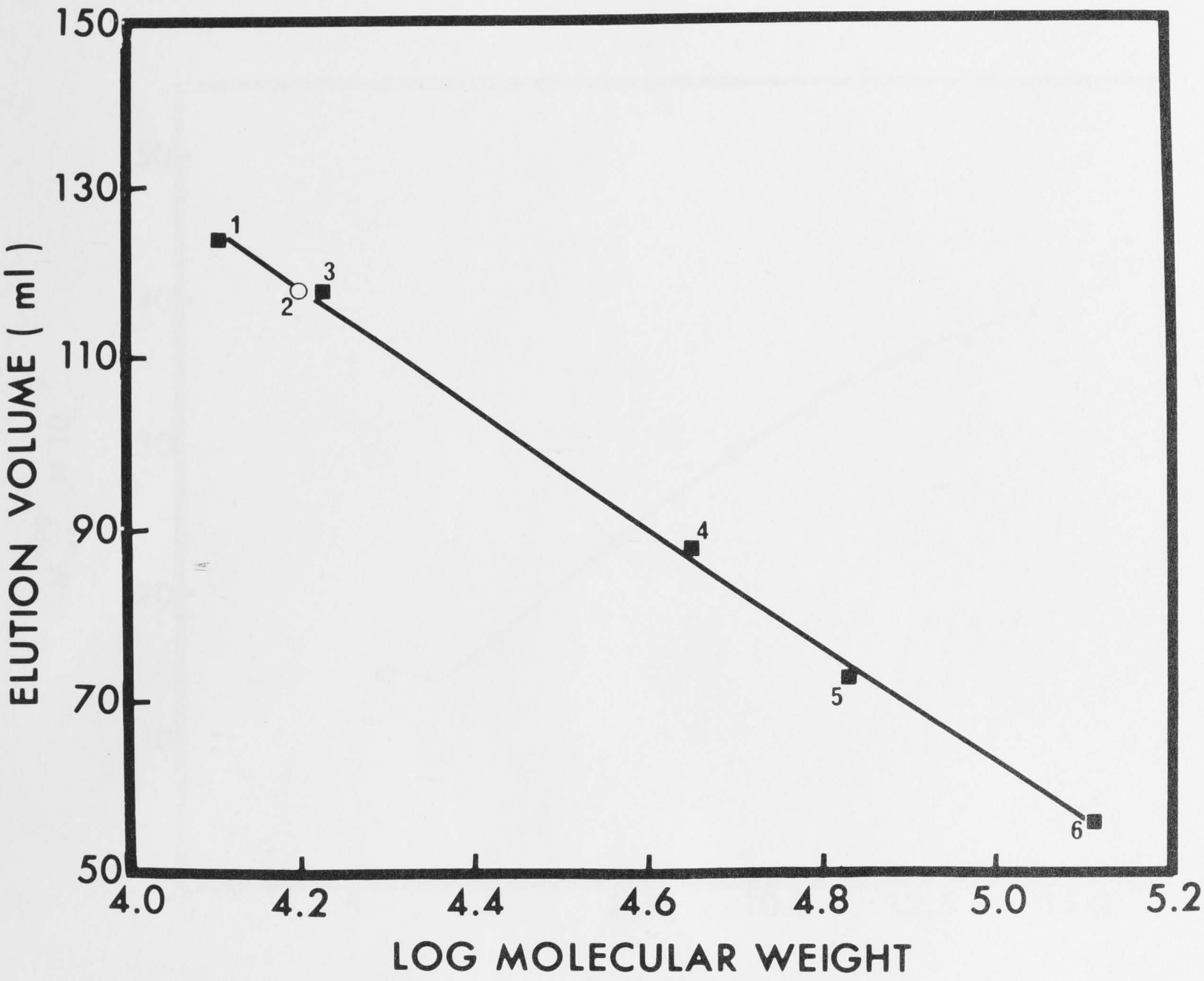


FIGURE 3.11 Estimation of the molecular weight of chorismate lyase by gel filtration on Sephadex G-100. The proteins used, together with their molecular weights, are shown below :

1.	ribonuclease	12,500
2.	chorismate lyase	15,800
3.	myoglobin	17,000
4.	ovalbumin	45,000
5.	serum albumin monomer	67,000
6.	serum albumin dimer	134,000

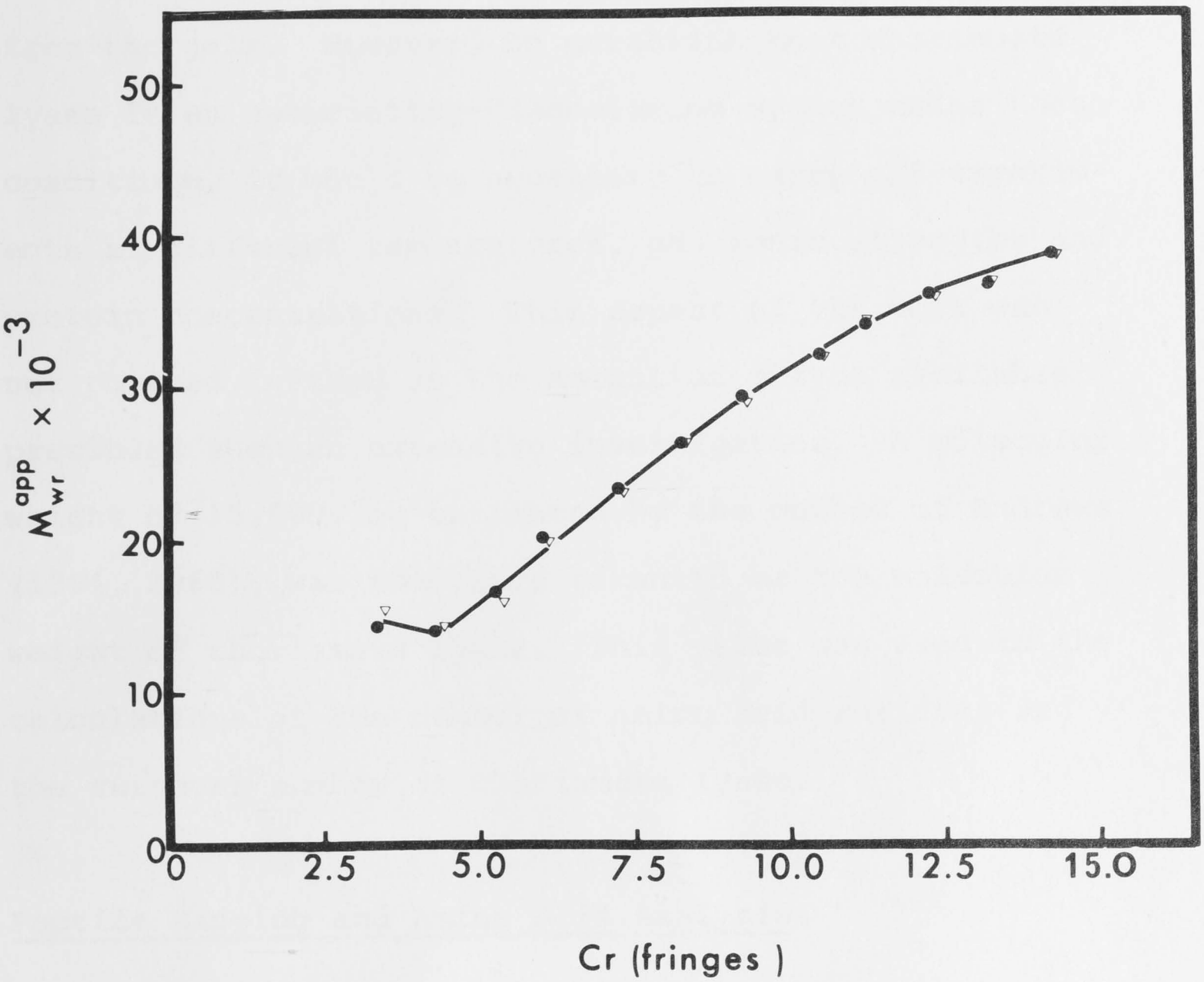


FIGURE 3.12 Estimation of the molecular weight of chorismate lyase by equilibrium centrifugation. The results are from two different estimations and are plotted together. Cr is the concentration of protein measured by displacement fringes, and extends over a range from ∿1 mg per ml at the meniscus to ∿4 mg per ml at the base of the cell. M_{wr}^{app} is the approximate molecular weight.

second conclusion is in accord with the data obtained from the gels. However, to establish that chorismate lyase is an associating-dissociating system under these conditions, it would be necessary to carry out experiments at different temperatures, pH, ionic strengths and protein concentrations. This aspect of the work was not pursued further as the amount of enzyme available precluded such an extensive investigation. A molecular weight of 15,800, as estimated by the method of Andrews (1964, 1965), was therefore taken to be the molecular weight of chorismate lyase. This value was used in the calculations of the number of amino acid residues and the turnover number of chorismate lyase.

Peptide Mapping and Amino Acid Analysis.

Conventional peptide mapping procedures were initially used. These involve a two dimensional separation on paper of a tryptic digest of the protein. Although these methods usually permit clear separation of the peptides into discrete spots, this was not the case in either of the solvent systems used, (Figure 3.13 and 3.14). Therefore, the dansylation technique employed by Atherton and Thomson (1969) was used to determine if separation of the 'dansylated' peptides could be obtained. This method has many advantages in comparison with conventional peptide methods, the most important of which is the small quantity

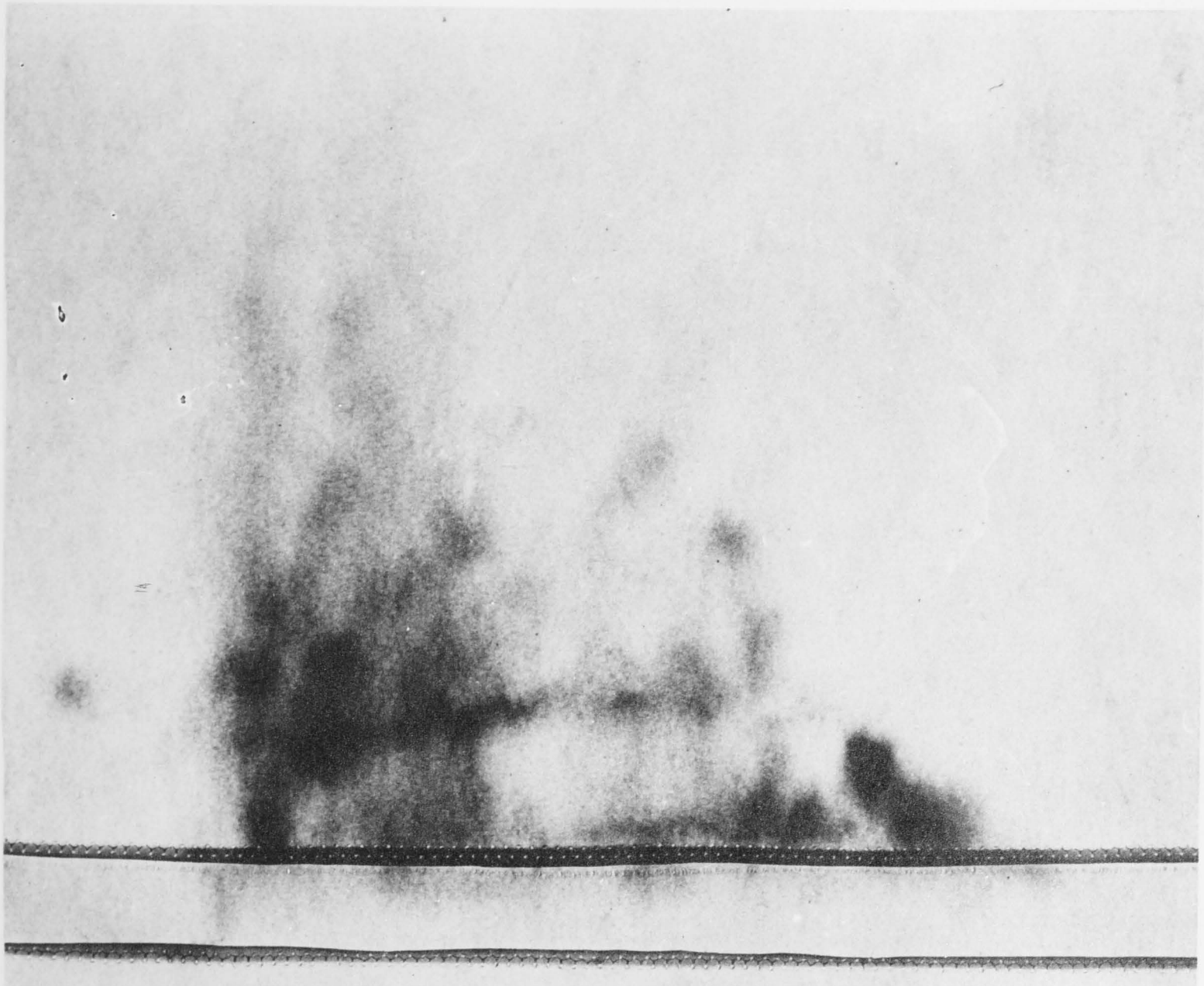


FIGURE 3.13 Peptide map of chorismate lyase from E. coli.

First dimension: Electrophoresis in pyridine acetate pH 4.7, 50 volts per cm for 2 h.

Second dimension: Chromatography in butanol:acetic acid:water (4:1:5, by vol., top phase). The map was stained with ninhydrin to show the peptides present.

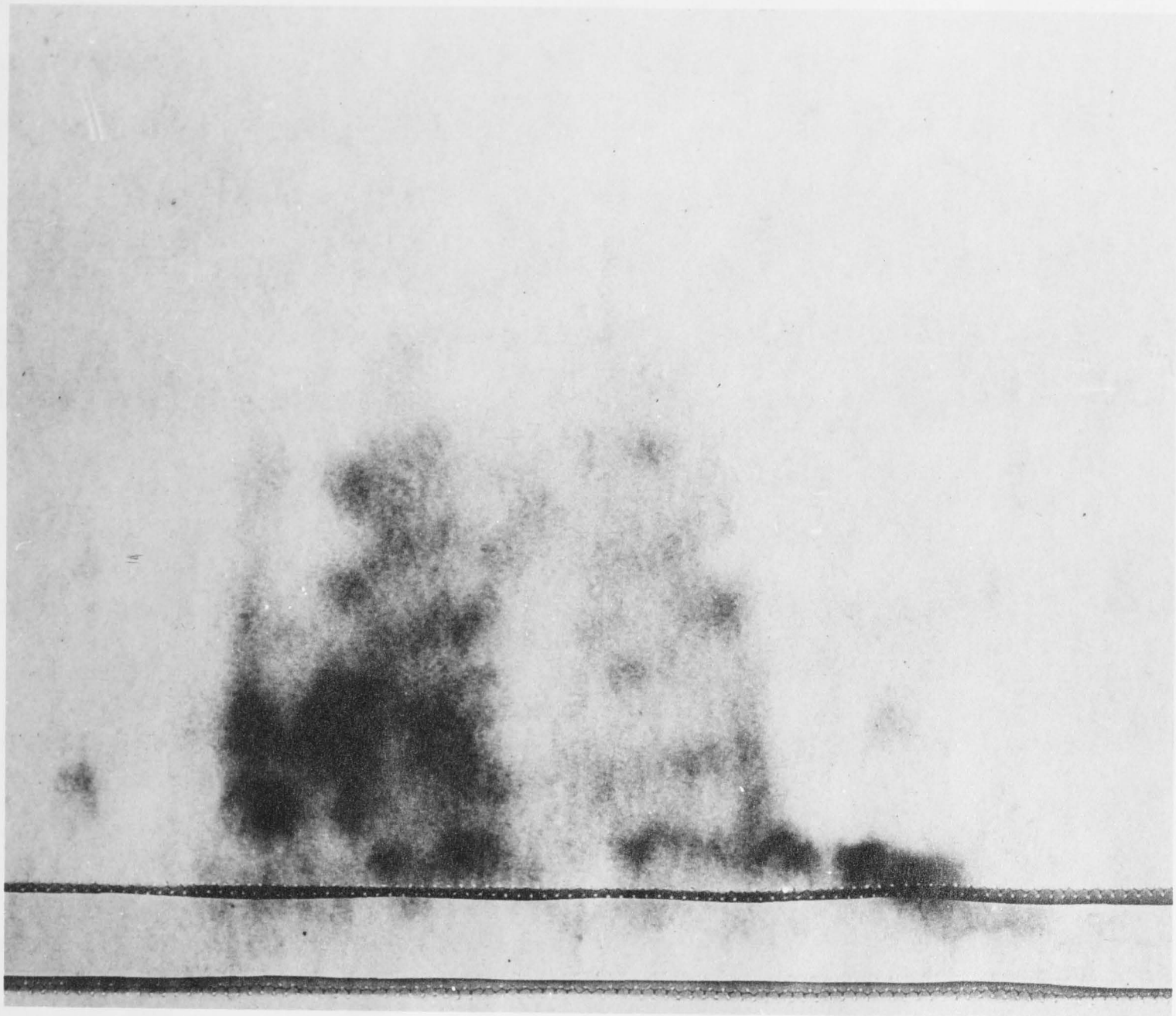


FIGURE 3.14 Peptide map of chorismate lyase from E. coli.

First dimension: Electrophoresis in pyridine acetate pH 4.7, 50 volts per cm for 2 h.

Second dimension: Chromatography in pyridine:isoamyl alcohol:water (7:7:6, by vol.). The map was stained with ninhydrin to show the presence of peptides.

of material that is required for each map. Another advantage is that the protein is made more hydrophobic which enables the peptides to be separated with organic solvents. There is non-specific staining of peptides when ninhydrin staining procedures are used, compared to the use of dansylated peptides which are each chemically reacted with the reagent before the map is developed. The results indicated that tryptic digestion gives rise to approximately 14 to 18 peptides (Figure 3.15).

Some criticism of the 'dansylated' method may be mentioned, in that very close to the origin it is difficult to determine the absolute number of peptides, as there appears to be a considerable amount of fluorescent material. To the naked eye, this area contains more discrete spots than is indicated by the photograph, so a diagram of the same chromatogram is included (Figure 3.16). In comparison to the number of peptides seen in the 'dansylated' peptide map, the number of peptides that theoretically are obtained from the amino acid composition (Table VI) is approximately 13. This estimation of the number of peptides is determined from the number of lysine and arginine residues calculated for a molecular weight of 15,800 for chorismate lyase. Since chorismate lyase is a single polypeptide chain, then each of the residues lysine and arginine would be present in a unique sequence. The total number of tryptic peptides on the fingerprints would be given by:

Total number of lysine and arginine residues plus one.

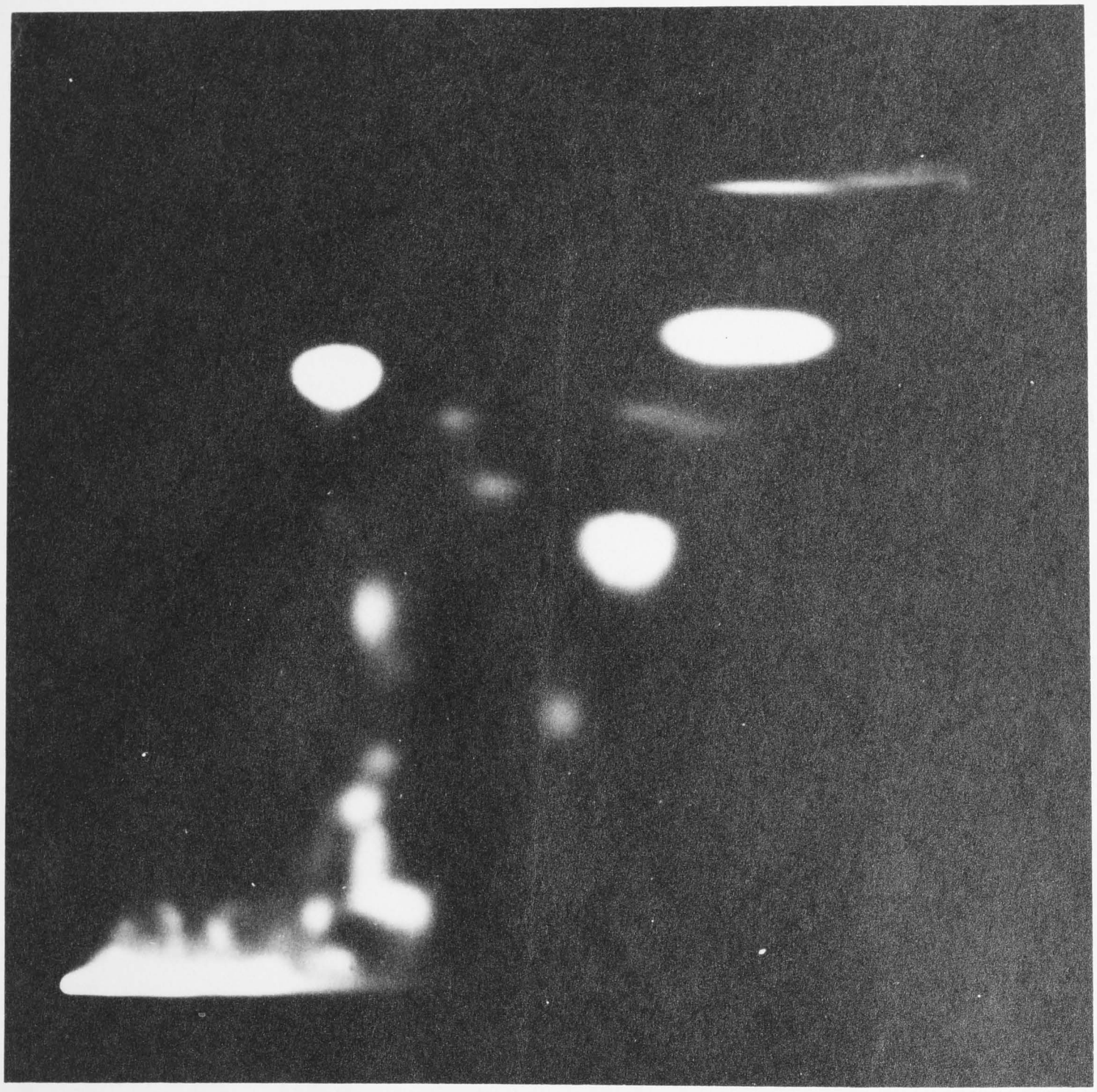


FIGURE 3.15 Photograph of a 'dansylated' peptide map of chorismate lyase, taken through a yellow filter. The origin is in the bottom left hand corner.

First dimension: Developed in methyl acetate:propan-2-ol:aqueous ammonia (9:7:4, by vol.).

Second dimension: Developed in chloroform:95% ethanol:acetic acid (38:4:3, by vol.).

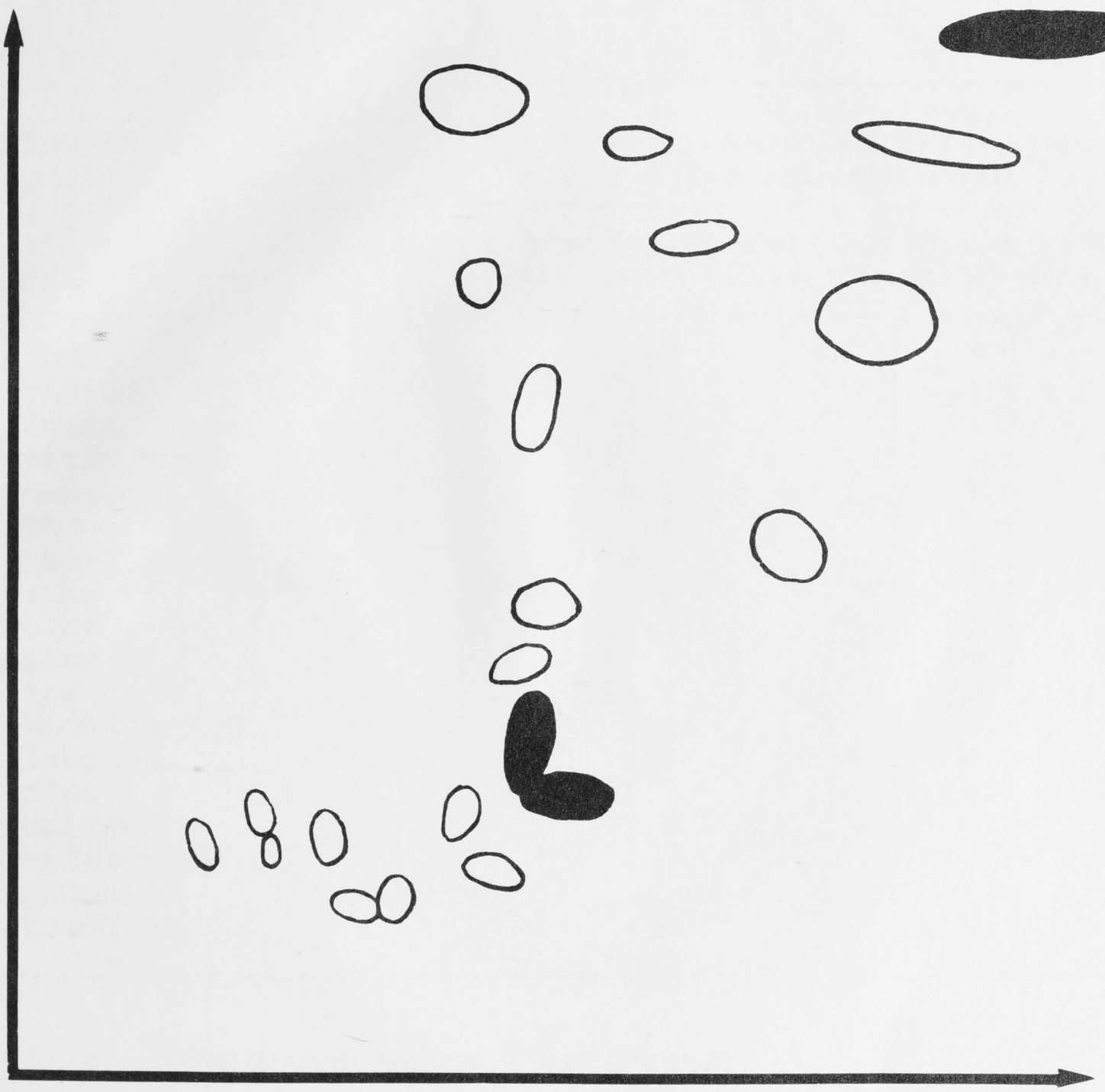


FIGURE 3.16 A diagrammatic representation of Figure 3.15, with the by-products of the reagent indicated by shaded spots. Very faint spots have been omitted.

TABLE VI. Amino Acid Composition of Chorismate Lyase.

Amino Acid	Number of Residues calculated for a molecular weight of 15,800.	
	Average of Two Determinations	Performic acid oxidised sample.
Lysine	6.9	7.9
Histidine	1.3	0.9
Arginine	3.8	3.4
Aspartic Acid	12.4	13.5
Threonine	6.6	7.0
Serine	6.6	7.0
Glutamic acid	13.5	14.2
Proline	6.8	5.6
Glycine	17.5	18.4
Alanine	17.1	18.2
Valine	11.3	11.3
Methionine	3.1	3.1[b]
Isoleucine	6.2	6.2
Leucine	11.2	10.7
Tyrosine[c]	2.0	-
Phenylalanine	3.3	1.9
Tryptophan[a]	-	-
Cysteine [d]		0.6

a. Tryptophan was not estimated.

b. Methionine was estimated as methionine sulphone and d. cysteine was estimated as cysteic acid in the performic acid oxidised sample.

c. Tyrosine was destroyed after performic acid oxidation.

It is to be noted in the amino acid analysis that there is only one residue of cysteine per molecule of chorismate lyase. The lack of involvement of the -SH group in the activity of the enzyme is confirmed later in this section when the activity of the enzyme is shown not to be affected by the sulphydryl reagents, iodoacetamide and N-ethylmaleimide. The larger number of peptides obtained on the peptide map could be explained by artifacts either arising from the tryptic digest or from excess fluorescence on the chromatogram. This latter comment would require that analysis of the 'dansylated' peptides be carried out in order to determine that all fluorescent spots were peptides derived from chorismate lyase.

Identification of the Reaction Products.

The conversion of chorismate to 4-hydroxybenzoate was established by Gibson and Gibson (1962). The establishment of pyruvate as the second reaction product is shown in Figure 3.17. The chromatogram of the 2,4 dinitrophenylhydrazone derivative of pyruvate is obtained from the reaction mixture of chorismate and chorismate lyase. This derivative co-chromatographed with the synthetic 2,4 dinitrophenylhydrazone derivative of pyruvate. Further, the evidence of the coupled enzyme system,

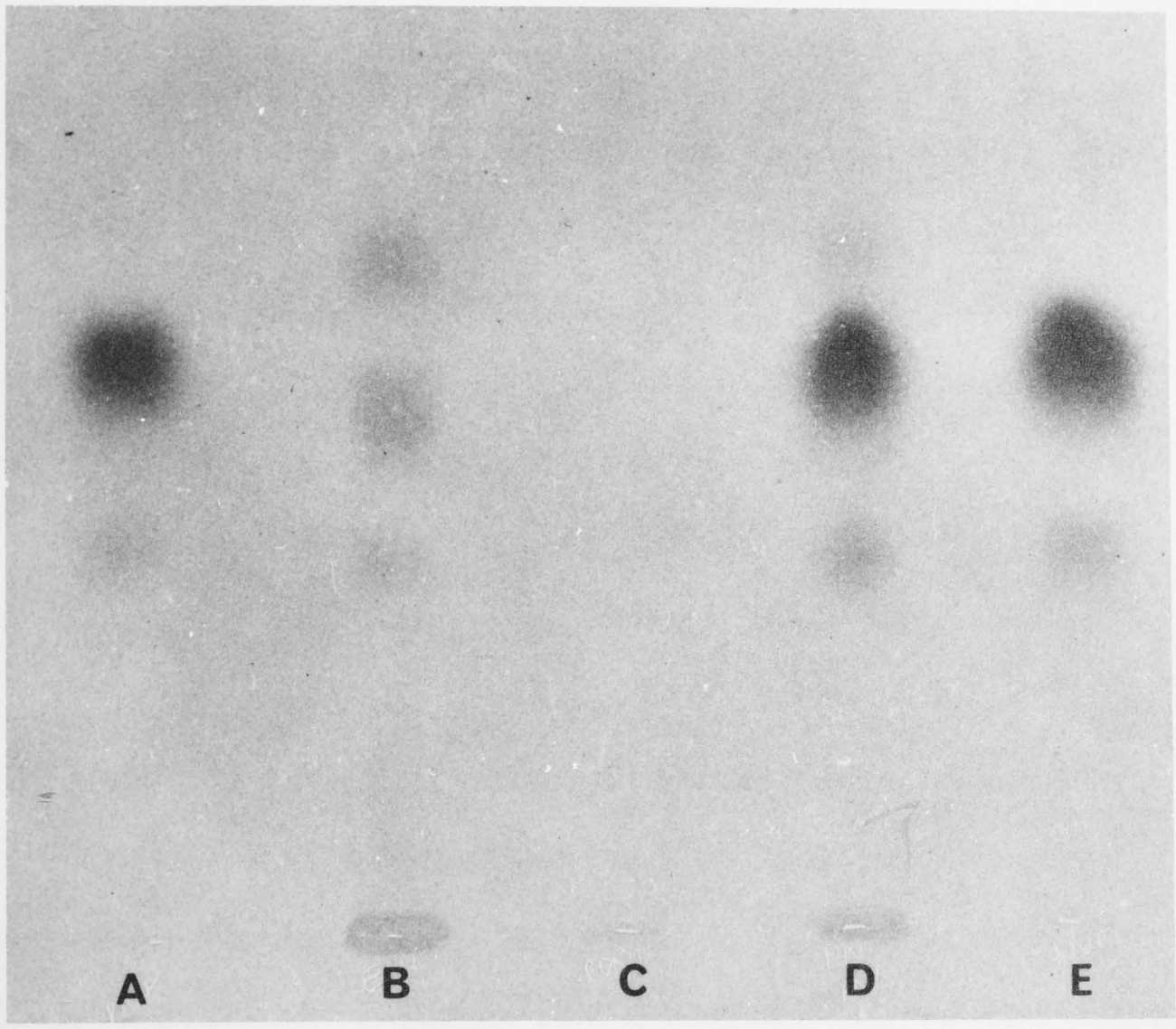


FIGURE 3.17 Chromatography on silica-gel plates of the 2,4-dinitrophenylhydrazone derivative of pyruvate.

A. 2,4-dinitrophenylhydrazone synthetic derivative of pyruvate.

B. reaction mixture treated with 2,4-dinitrophenylhydrazine.

C. control blank for the reaction treated with 2,4-dinitrophenyl-hydrazine.

D. reaction mixture co-chromatographed with the 2,4-dinitrophenylhydrazone synthetic derivative.

E. 2,4-dinitrophenylhydrazone synthetic derivative of pyruvate.

lactate dehydrogenase and NADH, also establishes one of the products of the reaction as pyruvate. In this connection it should be mentioned that 4-hydroxybenzoate is not a substrate for the reaction. The only other product must therefore be a ketone product.

Stoichiometry of the Reaction.

The amount of each product formed from chorismate by chorismate lyase was determined by the normal spectrophotometric methods. 4-Hydroxybenzoate and pyruvate are formed in equimolar amounts (Table VII). Since the amount of pyruvate formed is equivalent to the amount of 4-hydroxybenzoate formed from chorismate, initial rates of the reaction were thereafter measured using the pyruvate assay.

Equilibrium Constant Determination.

The equilibrium constant for the reaction was determined using the coupled enzyme system, and calculated to be 2.7×10^{-5}M from two experiments.

Time Course of the Reaction.

The rate of the reaction converting chorismate to 4-hydroxybenzoate and pyruvate begins to fall off after 10 min, and equilibrium is reached after 1 h (Figure 3.18).

TABLE VII. Determination of the Stoichiometry of the Reaction.

Time (min)	Concentration of Pyruvate formed (μmoles per ml)	Concentration of 4-hydroxybenzoate formed (μmoles per ml)
0	0	0
10	0.015	0.10
20	0.047	0.040
30	0.078	0.060
60	0.141	0.154
90	0.203	0.211
120	0.287	0.298
150	0.328	0.315
180	0.323	0.320

The reaction mixture consisted of 4.0 mM chorismate, 600 μg chorismate lyase, 5 μg lactate dehydrogenase, 0.1 mM NADH in a total volume of 1.0 ml. The appropriate blank was used and estimation of pyruvate and 4-hydroxybenzoate was carried out as described in MATERIALS AND METHODS.

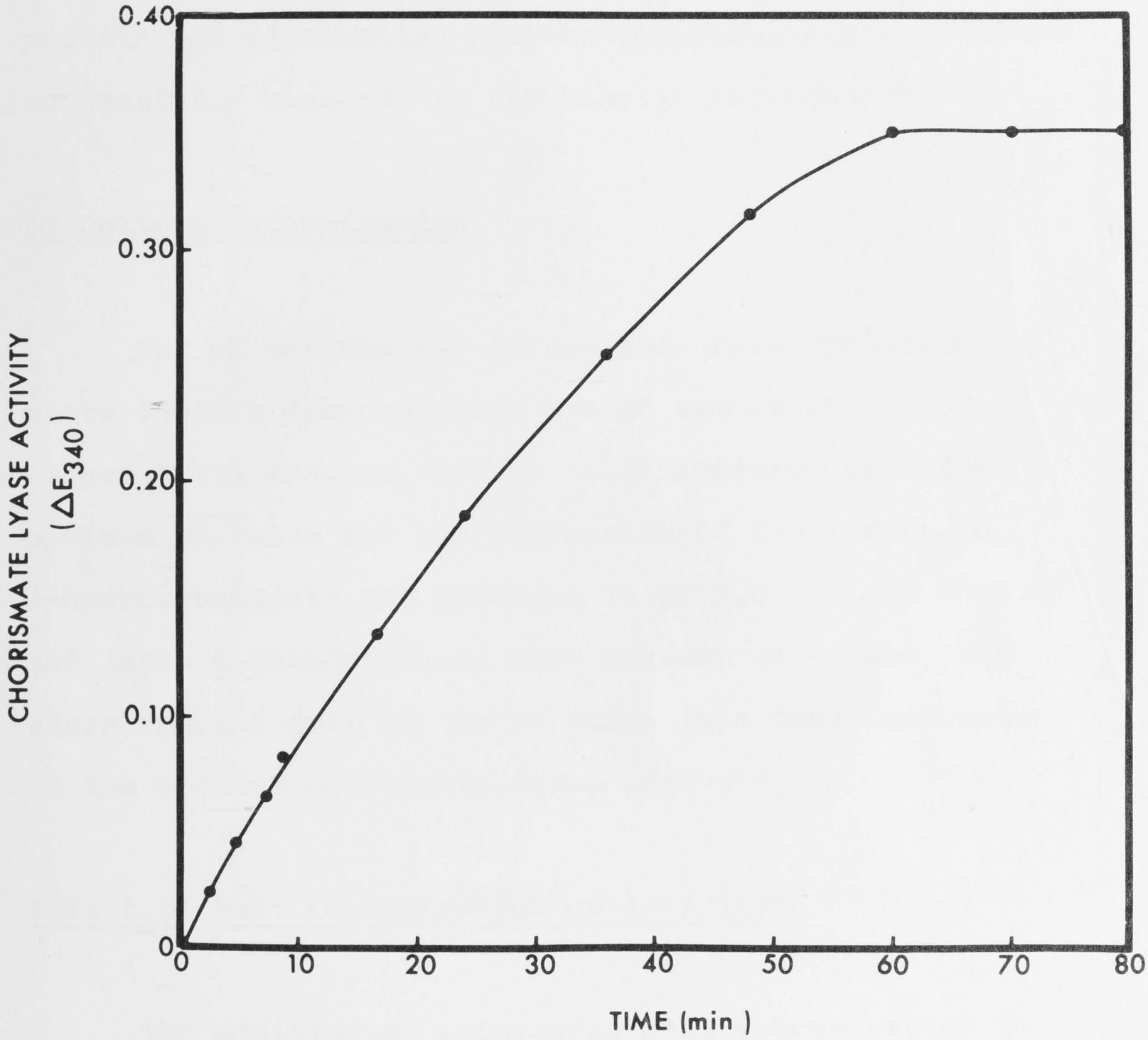


FIGURE 3.18 Time course of the reaction catalysed by chorismate lyase, as measured by the NADH/lactate dehydrogenase assay --- see MATERIALS AND METHODS.

When the substrate concentration was 1 mM or higher, and the enzyme concentration was relatively high (200 µg protein per ml reaction mixture), initial rates could be successfully measured in the kinetic experiments.

pH Optimum Determination.

The pH optimum was determined under conditions where lactate dehydrogenase was in excess at all pH values. The results (Figure 3.19) indicate that the optimum pH value for the conversion of chorismate to 4-hydroxybenzoate and pyruvate is pH 8.0. Since NADH and lactate dehydrogenase were present in excess, the sharp rise and fall of the pH curve is a true indication of the optimum chorismate lyase activity.

Effect of Salt Concentration on Activity.

The addition of increasing salt concentration to standard reaction mixtures resulted in an initial marked activation of the enzyme, and this was then followed by its inhibition. Optimum activation was obtained at a salt concentration of 0.3M (Figure 3.20). The results may be compared with those obtained earlier (Section 3.1), where an increase and decrease in enzymic activity was observed when the ionic strength of the buffer was raised and lowered respectively.

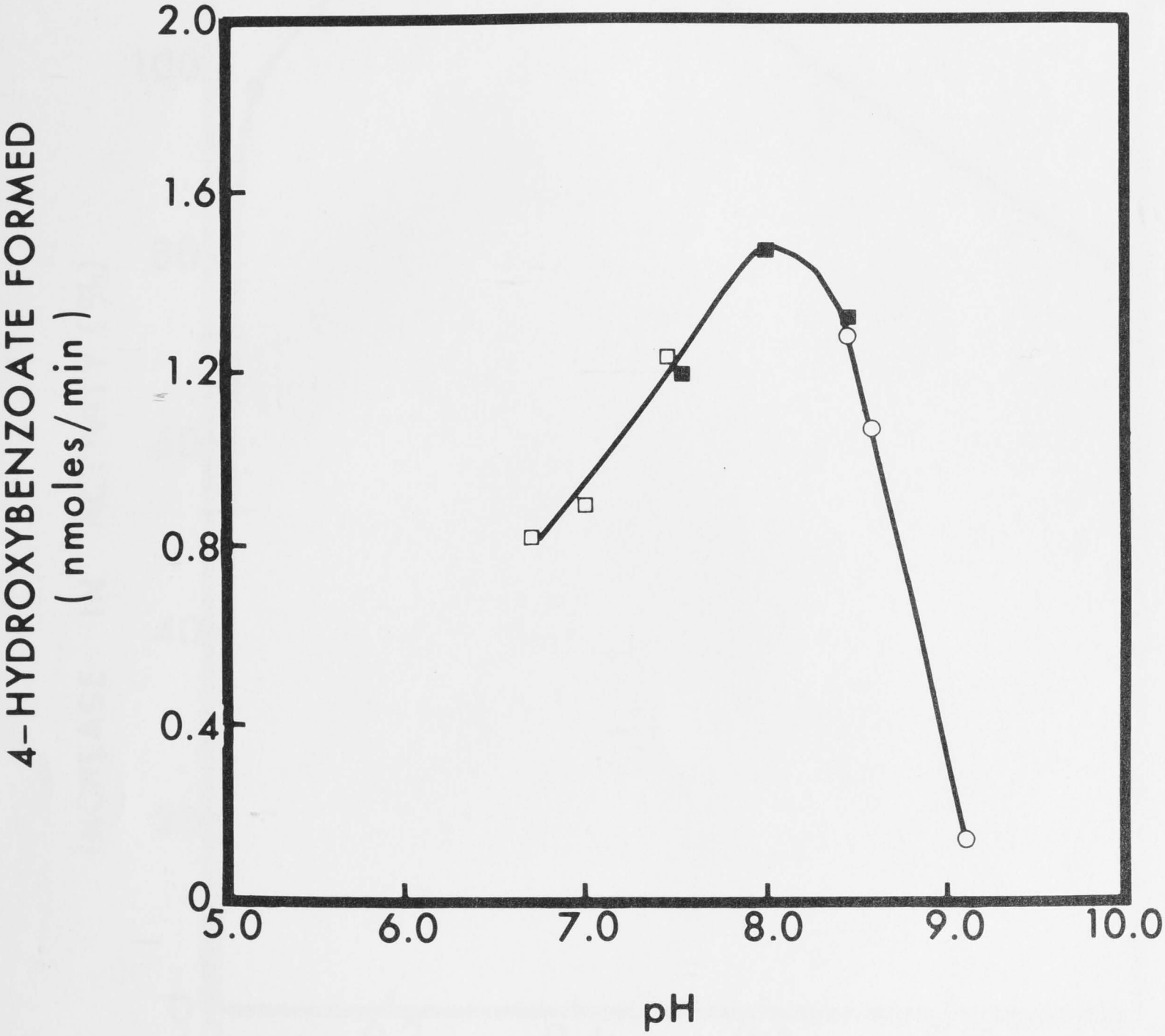


FIGURE 3.19 The effect of pH on the velocity of enzymic activity, as measured by the NADH/lactate dehydrogenase assay --- see MATERIALS AND METHODS.

□—□, TES-TRIS Buffer, pH 6.5 - pH 7.5;

■—■, TRIS-HCl Buffer, pH 7.5 - pH 8.5;

o—o, TRIS-Glycine Buffer, pH 8.5 - pH 10.0.

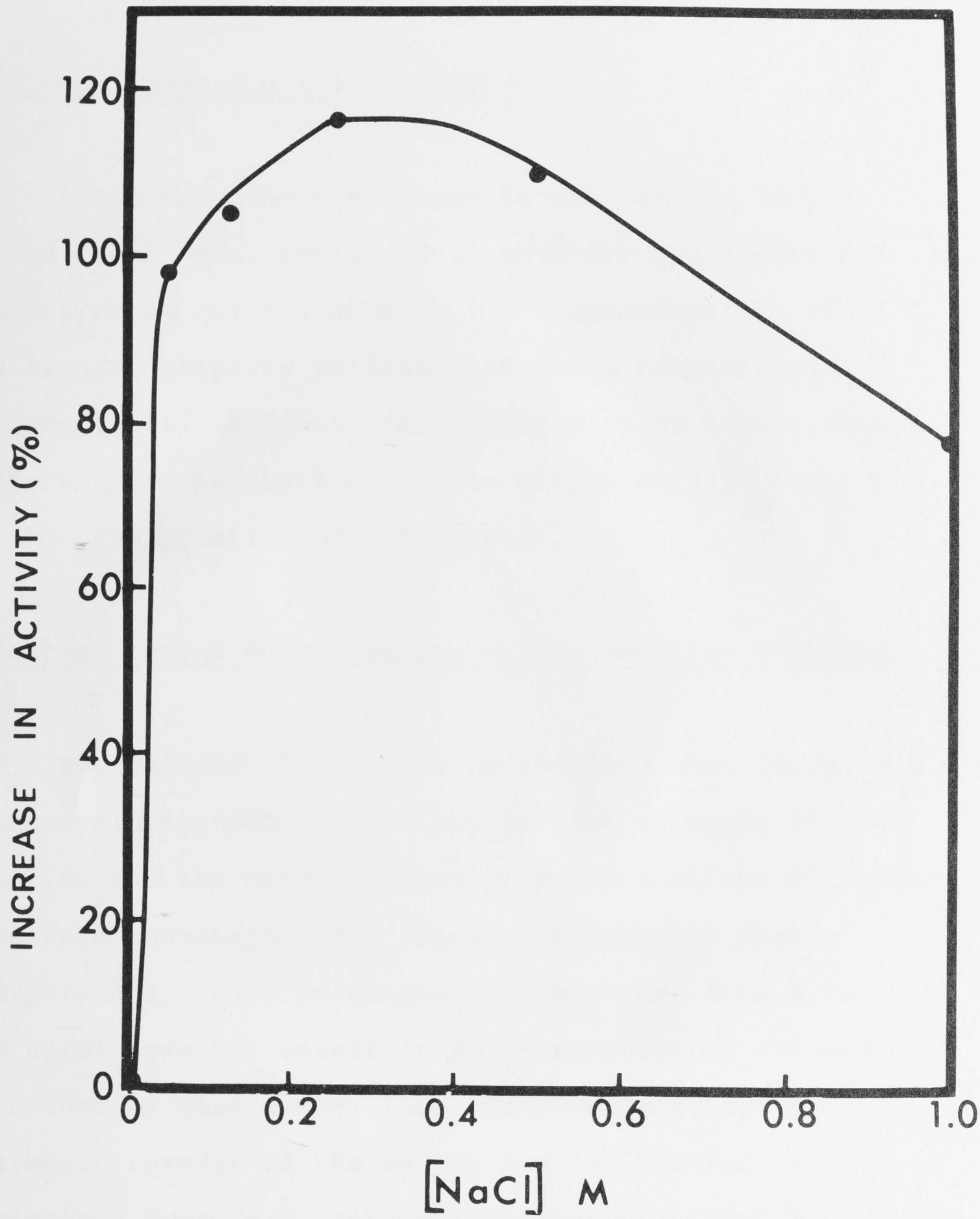


FIGURE 3.20 The effect of increasing salt concentration on enzymic activity, expressed as the percentage increase in activity above the normal level. The enzymic activity was measured by the NADH/ lactate dehydrogenase assay --- see MATERIALS AND METHODS.

Effect of Temperature on Activity.

After exposing the enzyme to the various temperatures for 30 min, the residual activity was determined. The enzyme is quite stable at low temperatures up to 37°C, and is also slightly activated at these temperatures (Figure 3.21). Activity is rapidly lost at higher temperatures, and incubation of the enzyme at 45°C resulted in the precipitation of the protein.

Effect of Enzyme Concentration on the Reaction Velocity.

The results of Figure 3.24 indicate that there is a linear relationship between the initial velocity of the reaction and the concentration of protein at two different substrate concentrations. These data suggest that a forty-fold increase in enzyme concentration from 5 to 200 µg/ml does not result in polymerization of the enzyme to a less or more active form, or alternatively that polymeric form(s) of the enzyme possess the same specific activity. Therefore, no complications arise in the kinetic investigations.

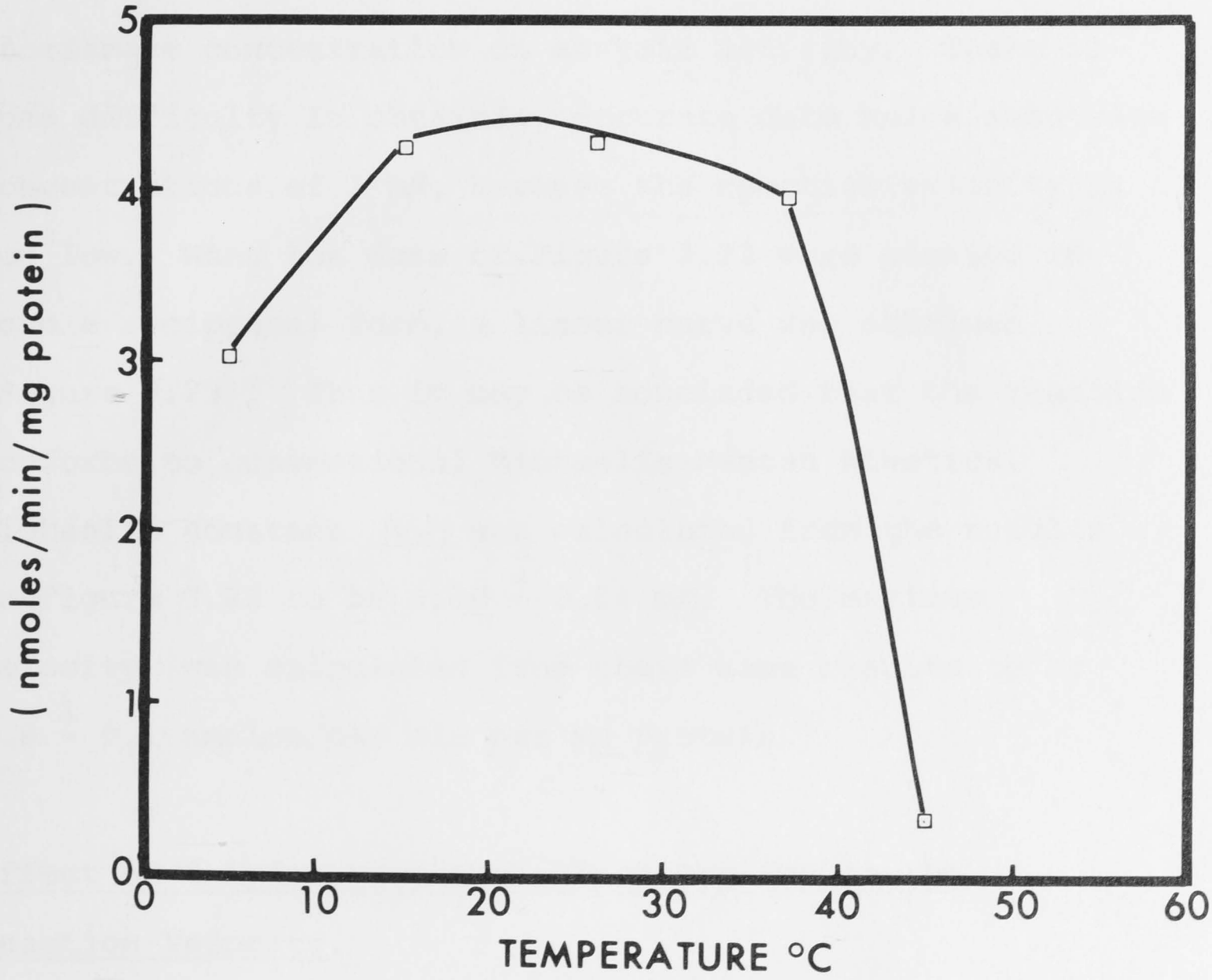


FIGURE 3.21 The effect of temperature on chorismate lyase activity. Chorismate lyase was subjected to the various temperatures before determining the initial rates by the NADH/lactate dehydrogenase assay --- see MATERIALS AND METHODS.

Effect of Chorismate Concentration on the Reaction Velocity.

Figures 3.22 and 3.23 show the effect of varying chorismate concentration on enzymic activity. There is some difficulty in obtaining accurate data below substrate concentrations of 1 mM, because the reaction velocity is too low. When the data of Figure 3.22 were plotted in double reciprocal form, a linear curve was obtained (Figure 3.23). Thus it may be concluded that the reaction conforms to conventional Michaelis-Menten kinetics. Michaelis constant (Km) was calculated from the results in Figure 3.23 to be 0.10 ± 0.04 mM. The maximum velocity was calculated from these same results to be 6.8 ± 0.1 nmoles/per min per mg protein.

Effect of 4-Hydroxybenzoate Concentration on the Reaction Velocity.

The linear competitive inhibition of the enzyme reaction by 4-hydroxybenzoate is illustrated in Figure 3.25, and it will be noted that considerable inhibition is observed in the presence of relatively low concentrations (0.05mM and 0.1 mM) of 4-hydroxybenzoate. The data were fitted to equation (2) and a value of 6 ± 2 μM was obtained for the inhibition constant (Ki). The ratio of Km for chorismate: Ki for 4-hydroxybenzoate was calculated to be 17.8 ± 1.0 from which it may be

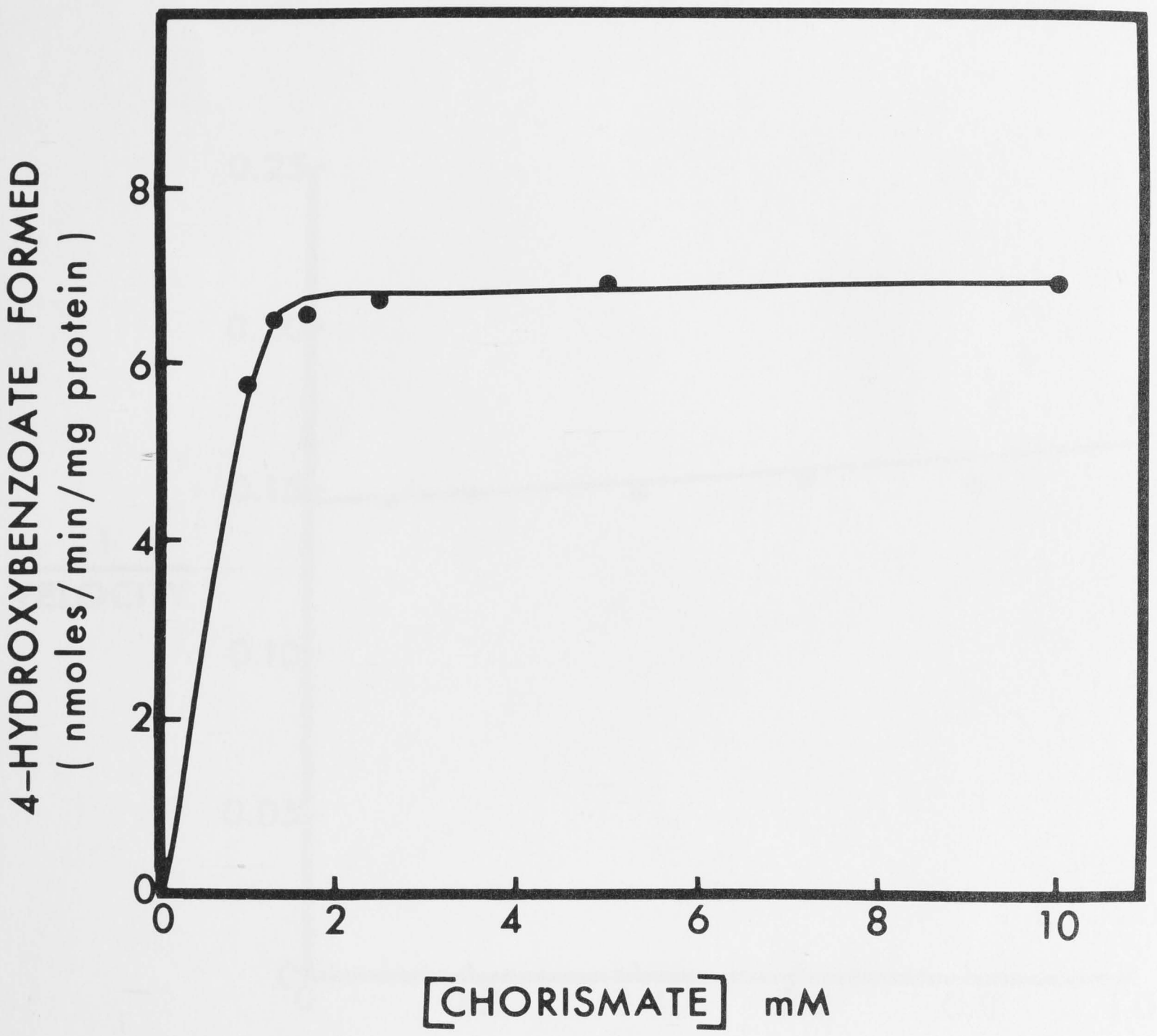


FIGURE 3.22 The effect of increasing substrate concentration plotted against the initial rates of the reaction, as measured by the NADH/lactate dehydrogenase assay --- see MATERIALS AND METHODS.

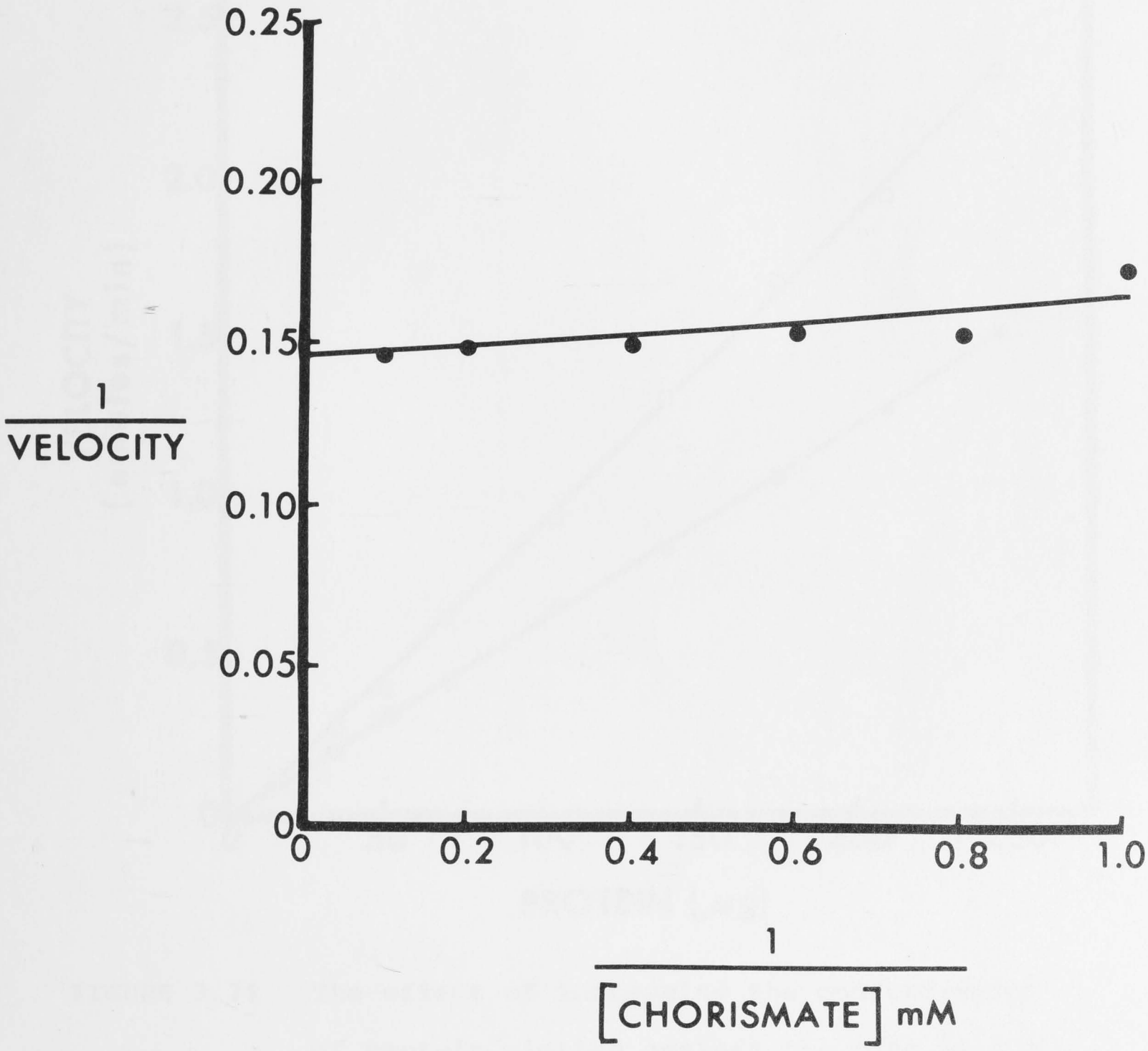


FIGURE 3.23 Double reciprocal plot using the same values as in Figure 3.22. The Km was calculated to be 0.10 ± 0.04 mM.

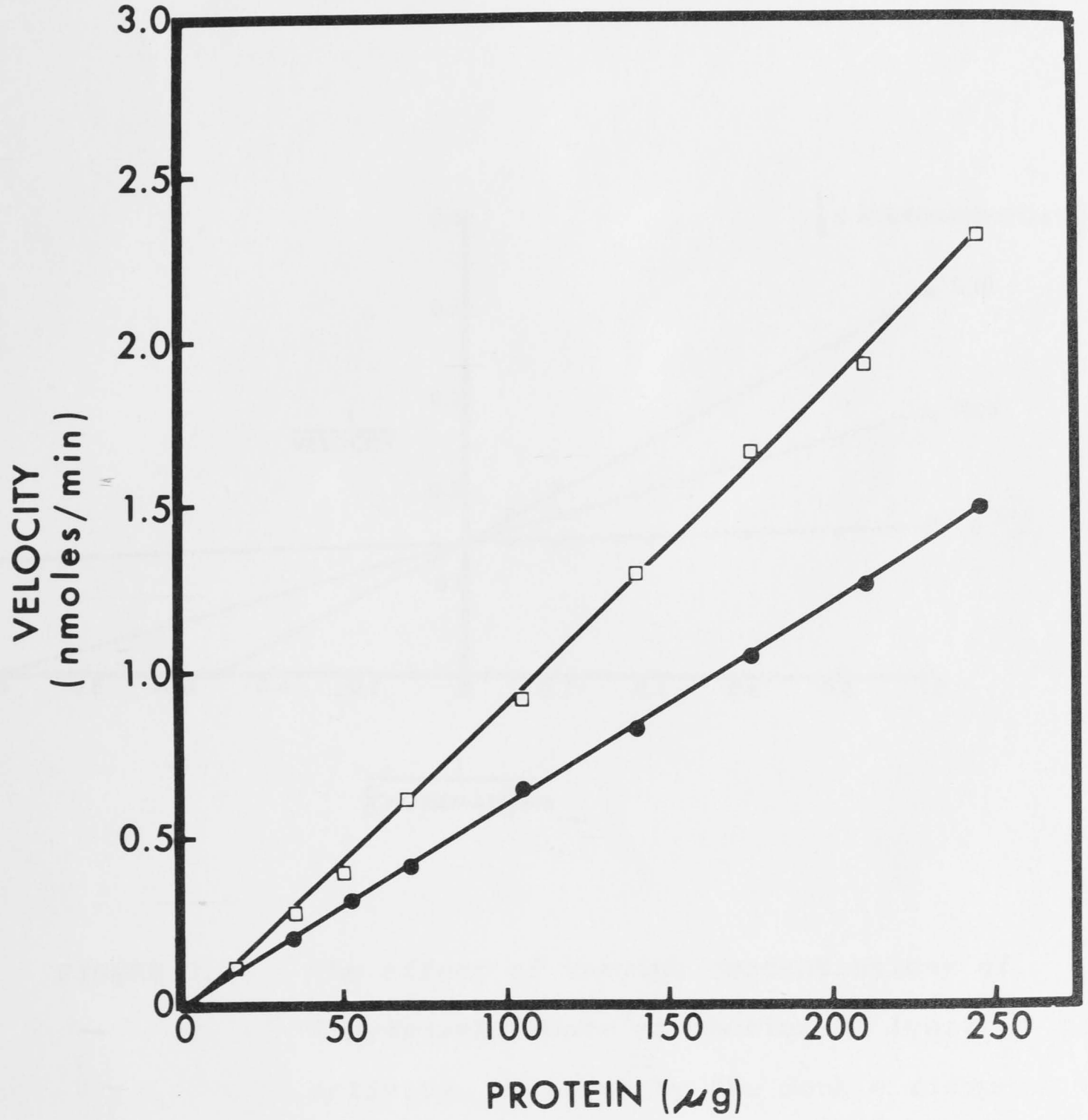


FIGURE 3.24 The effect of increasing the concentration of protein plotted against the rate of the reaction, as measured by the NADH/lactate dehydrogenase assay at two substrate concentrations :

■ — ■, 10 mM chorismate

● — ●, 1 mM chorismate

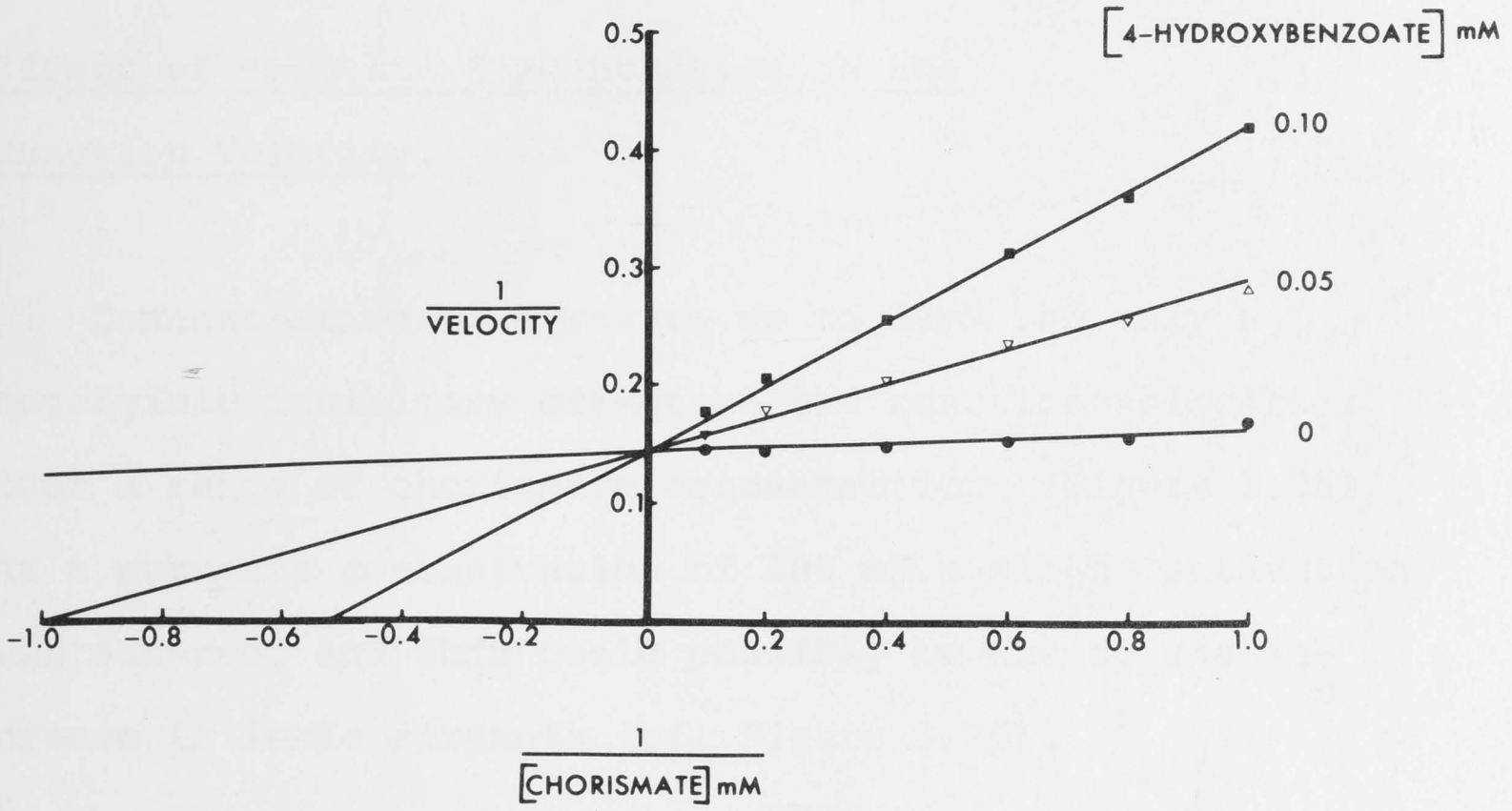


FIGURE 3.25 The effect of varying concentrations of 4-hydroxybenzoate on chorismate lyase activity, as shown by the double reciprocal plot. Initial rates for the reaction were measured by the NADH/lactate dehydrogenase assay. The Ki for 4-hydroxybenzoate was calculated to be 6 ± 2 μM.

concluded that 4-hydroxybenzoate is a potent inhibitor of the reaction. Since this product acts as a linear competitve inhibitor it follows that both chorismate and 4-hydroxybenzoate raect at the same site on the enzyme.

Effect of Pyruvate Concentration on the Reaction Velocity.

Concentration of pyruvate up to 20mM had only a negligible inhibitory effect on the reaction velocities over a range of chorismate concentrations (Figure 3.26). At a pyruvate concentration of 100 mM a slight activation was observed and this could possibly be due to the increase in ionic strength (cf. Figure 3.20).

Effect of Chemical Reagents on Enzymic Activity.

The effect of chemical modifying reagents on the activity of the enzyme is shown in Table Vlll. The range of concentrations that were used to observe effects on chorismate lyase activity was from 1 mM to 0.01 mM. Table Vlll shows the results of the various reagents used. Iodoacetamide and N-ethylmaleimide had no effect on chorismate lyase activity although not all sulphydryl groups react with iodoacetamide, they nevertheless will, if present, react with N-ethylmaleimide at a concentration

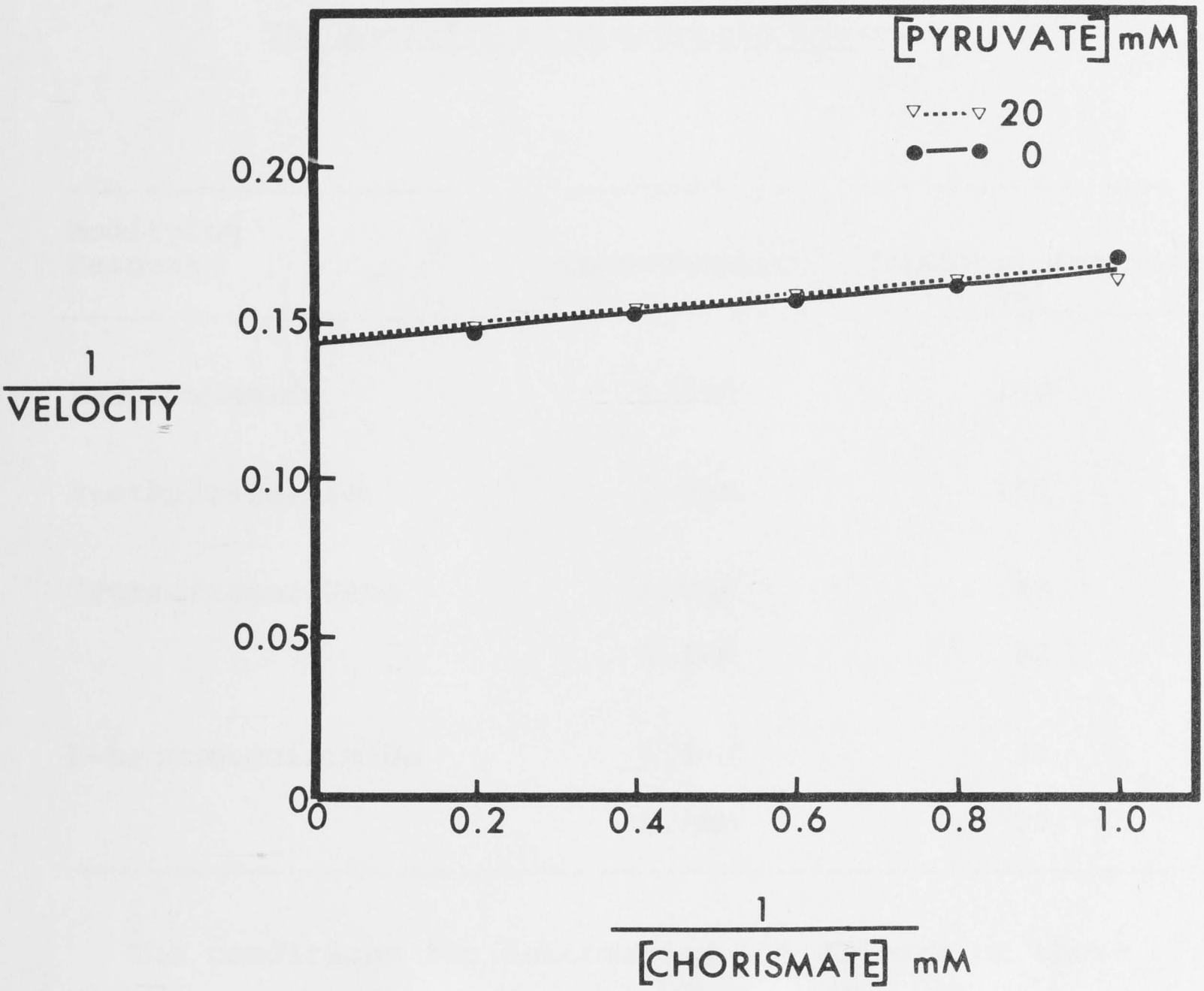


FIGURE 3.26 The effect of pyruvate, using a concentration of 20 mM, on chorismate lyase activity, as shown by the double reciprocal plot. Initial rates for the reaction were measured by the NADH/ lactate dehydrogenase assay.

TABLE VIII. Effect of Various Modifying Reagents on The Activity of Chorismate Lyase.

Modifying Reagent.	Concentration	Residual Activity (%)
Iodoacetamide	1.0mM	100
N-ethylmaleimide	1.0mM	100
Tetranitromethane	1.0mM	48
	0.1mM	92
N-bromosuccinimide	1.0mM	31
	0.1mM	96

The conditions for determining the effects of these reagents were the same as those for 4-hydroxybenzoate estimation as described in MATERIALS AND METHODS. The protein was pre-incubated for 30 min at 37°C in the presence of modifying reagents before assaying for activity.

of 1.0 mM. The chemical reagent 1.0 mM tetranitromethane is effective in destroying 52% of the activity while N-bromosuccinimide is effective in destroying 69% of the activity of chorismate lyase.

Effects of Analogues and Allosteric Modifiers on the Reaction.

Various compounds which could be considered as analogues of both chorismate and 4-hydroxybenzoate were used over the concentration range of 0.01 mM to 1.0 mM to determine whether they were able to function as inhibitors of chorismate lyase. The results are shown in Table IX. Those compounds which greatly affect chorismate lyase activity are 3,4-dihydroxybenzoate (1.0 mM) and 2,5-dihydroxybenzoate (0.1 mM) where the activity is reduced to zero at the concentrations used. These results suggest that the hydroxyl group is involved with the inhibition of the reaction. Further, 4-aminobenzoate has no inhibitory affect on the activity of the enzyme and a slight activation is observed with this compound. This result supports the requirement of an hydroxyl group for inhibition to take place.

Various other compounds that function as allosteric modifiers of other enzymes were also tested to determine if they were capable of influencing the catalytic activity of chorismate lyase. The results indicated that

TABLE IX. Inhibition and Activation of Chorismate Lyase by Various Substrate and Product Analogues.

Addition	Concentration	Relative Activity (%)
None	-	100
2,3-dihydroxybenzoic acid	1.0mM	100
phenylalanine	1.0mM	114
	0.1mM	100
anthranilic acid	1.0mM	100
tryptophan	1.0mM	100
salicylic acid	1.0mM	100
4-aminobenzoic acid	1.0mM	111
tyrosine	1.0mM	112
barium prephenate	1.0mM	100
3,4-dihydroxybenzoic acid	1.0mM	4
	0.1mM	72
	0.01mM	91
3-hydroxybenzoic acid	1.0mM	100
3-(1'-carboxyvinyloxy) benzoic acid	1.0mM	80
phenylacetic acid	1.0mM	100
phenyllactic acid	1.0mM	100
2,5-dihydroxybenzoic acid	0.1mM	0
	0.01mM	100

Compounds were added to the standard reaction mixture giving a final concentration as shown in the Table. Conditions for the assay of enzymic activity were the same as described in MATERIALS AND METHODS. Activity is expressed as a percentage of the activity of the enzyme with no additions.

fructose 1,6 diphosphate (1 mM), cyclic adenosine monophosphate (1 mM) and adenosine triphosphate (1 mM) and $MgCl_2$ (10 mM) neither activated or inhibited the enzyme.

DISCUSSION

As mentioned in Section 3.1, certain difficulties arise in the determination of the concentration of chorismate lyase in the cell. This aspect has therefore been ignored. However, since one of the primary aims of the work presented in this thesis was to determine if chorismate lyase possessed a high or low turnover number, the data has been calculated and presented in Table X. A comparison of other enzymes which convert chorismate as substrate has also been made. It is clear from the values obtained that chorismate mutase-prephenate dehydrogenase which is associated with the production of tyrosine, a major biosynthetic product, possesses two large turnover numbers (of the order of one and three thousand). This enzyme is a bifunctional enzyme and the values obtained indicate the importance of the pathway. Similarly, chorismate mutase-prephenate dehydratase which is involved with the production of phenylalanine and is also a bifunctional enzyme has high turnover numbers for both activities (of the order of three and two thousand). Anthranilate synthetase which converts chorismate to anthranilate and so to tryptophan, has a turnover number considerably lower than the other two enzymes mentioned above (three hundred). However, this value is

TABLE X. The Turnover Number for Enzymes from E. coli Associated with Chorismate

Enzyme	Turnover Number moles of substrate metabolized per min per catalytic unit
Chorismate lyase	.06
Anthranilate synthetase[a]	676
Chorismate mutase-prephenate dehydrogenase[b]	
1. mutase	800
2. dehydrogenase	2,700
Chorismate mutase-prephenate dehydratase[c]	
1. mutase	2,700
2. dehydratase	1,200

a) Data calculated from Baker and Crawford (1966) and Ito and Yanofsky (1969).

b) Data calculated from Koch et al. (1971).

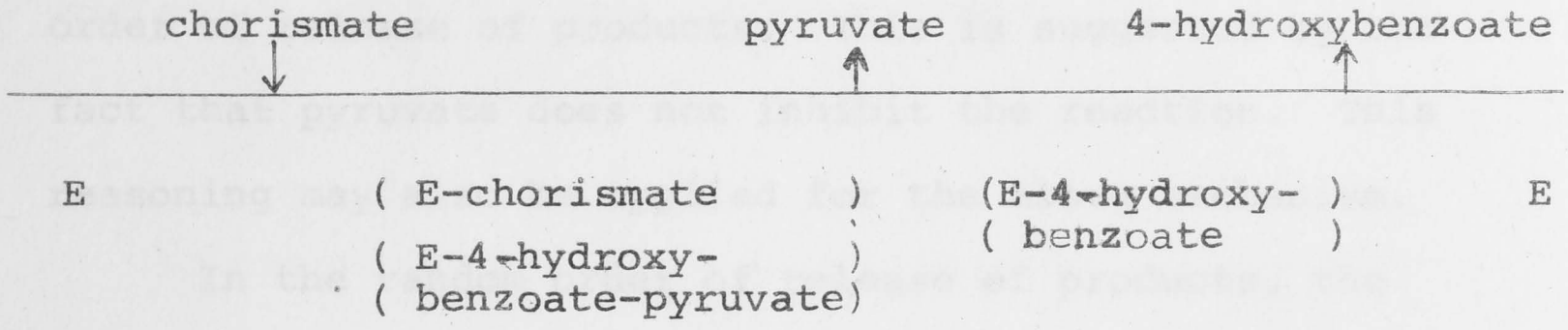
c) Data calculated from Davidson et al. (1972).

high compared to the turnover number for chorismate lyase. This number, of .06 is very low and is not unexpected because of (1) the role of 4-hydroxybenzoate in the cell's metabolism and (2) the pathway involved in the biosynthesis of ubiquinone is a minor one compared to the pathways involved in the production of the amino acids.

With the identification and formulation of the reaction products in equimolar amounts and the isolation of a homogeneous protein, the enzyme chorismate lyase, was therefore suitable for mechanistic studies. The discussion of the possible mechanism of the reaction is limited to a discussion of qualitative data only because no inhibition by pyruvate was observed.

Both steady-state kinetics and Michaelis kinetics assume that no inhibition is observed by the products or by the substrate. However, in discussing the possible mechanism of the reaction, steady-state kinetics is assumed, as it is the easier of the two to discuss when drawing conclusions about alternative mechanisms of the reaction.

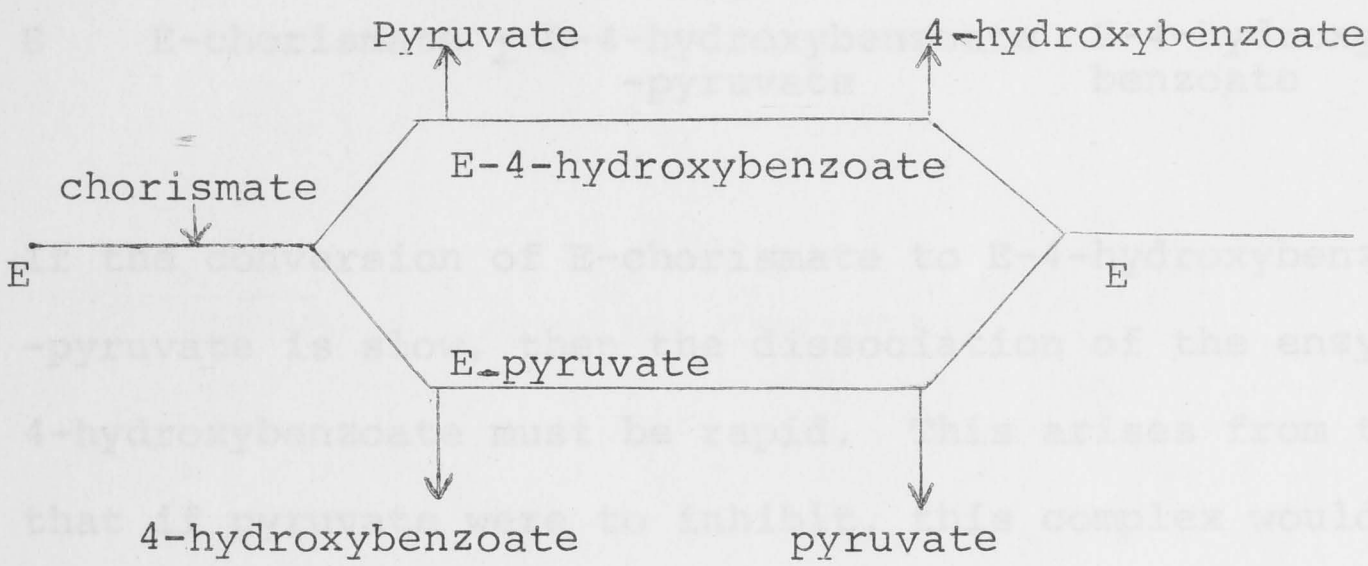
From the information obtained three possible mechanisms are proposed. The first mechanism shown below assumes steady-state kinetics and is ordered.



Initially, in this mechanism chorismate binds quite strongly to the Enzyme. If an ordered mechanism exists, pyruvate must be released first from the Enzyme-chorismate complex, thus leaving the Enzyme-4-hydroxybenzoate complex. This complex dissociates very slowly as is indicated by the Ki for 4-hydroxybenzoate, (Ki = 0.006 $\pm$ 0.002 mM). It thus competitively inhibits the reaction. It is also possible in a steady-state that the release of pyruvate could also be a slow step. However, regardless of whether the pyruvate and/or the 4-hydroxybenzoate release is slow, the Enzyme-chorismate complex is competively inhibited by the formation of the Enzyme-4-hydroxybenzoate complex. This mechanism implies that the site on the Enzyme at which 4-hydroxybenzoate inhibits is the same site or very close to the site to which chorismate binds, as 4-hydroxybenzoate is a very effective inhibitor. If this were not so, then by altering the conformation of the protein slightly, the substrate would be able to bind as it does under normal conditions.

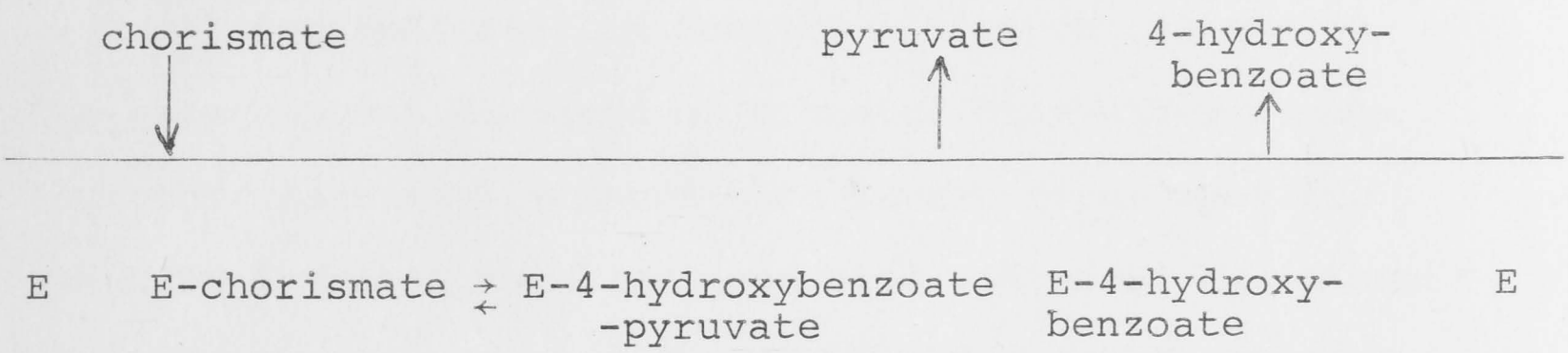
The second possible mechanism involves a random order of release of products. This is suggested by the fact that pyruvate does not inhibit the reaction. This reasoning may also be applied for the above mechanism.

In the random order of release of products, the following pattern is observed.



This means that on the one hand pyruvate may be released first or on the other, 4-hydroxybenzoate may be released first. However, in the former case, the E-4-hydroxybenzoate complex remains and this eventually will dissociate to form free enzyme and 4-hydroxybenzoate. In the latter case, 4-hydroxybenzoate is released from the complex initially. Thus in both cases 4-hydroxybenzoate is available to competitively inhibit the reaction. The one factor which must be considered in this mechanism, however, is that the breakdown of the enzyme substrate complex, to Enzyme-pyruvate-4-hydroxybenzoate, is the slow step. All other steps are fast and therefore permit this type of mechanism.

A third possible mechanism, similar to the first mechanism but yet allows pyruvate not to inhibit, is shown below.



If the conversion of E-chorismate to E-4-hydroxybenzoate -pyruvate is slow, then the dissociation of the enzyme bound 4-hydroxybenzoate must be rapid. This arises from the fact that if pyruvate were to inhibit, this complex would have to exist for some time in order to build up the concentration to a reasonable level.

These possible mechanisms are only able to be drawn from the data that has been obtained so far. However, more information is required about pyruvate in order to distinguish whether the machanism is ordered or random and which are the fast and slow steps in the dissociation of enzyme bound intermediates.

SECTION 4. GENERAL DISCUSSION.

GENERAL DISCUSSION.

It was indicated in Section 1 that chorismate is the branch-point compound of aromatic biosynthesis, and that the reaction discussed here is the first specific reaction involved in ubiquinone biosynthesis. In attempting to draw some conclusions about the possible role of chorismate lyase in the ubiquinone pathway and in more general terms, its relationship to the aromatic pathway, it must be remembered that ubiquinone is not a major product of aromatic bio-synthesis. This is in direct contrast to the amino acids.

Chorismate lyase has been shown to be a single polypeptide chain, having a molecular weight of 15,800. Although the protein was shown to be homogeneous by sedimentation equilibrium experiments and by polyacrylamide gel electrophoresis in sodium dodecyl sulphate, ultracentrifugation analysis indicated that aggregates were probably formed at higher protein concentrations (at approximately 4 mg/ml). It is in fact not uncommon for proteins to exist as aggregates in the pure state, where these aggregates represent a state of lower energy. For example, Crestfield et al. (1962) have shown that bovine pancreatic ribonuclease A associates quite readily, but this phenomenon appears to be an

artifact of lyophilisation. Dimers and higher oligomers of ribonuclease A are formed, and although these are stable at 25°C, they rapidly dissociate at 65°C. However, the specific activity of these aggregates is identical with the monomer.

Margoliash and Lustgarten (1962) have also demonstrated in studies on cytochrome c that the dimer crystallises together with the monomer. Among the proteins that are known to associate in the absence of disulphide links, may be found ribonuclease, cytochrome c, β-lactoglobulin, lysozyme and glutamic acid dehydrogenase (Reithel, 1963). To establish the presence of aggregates of chorismate lyase unequivocally, further experimentation would be required. If aggregates are present at lower protein concentrations, they are indistinguishable from the monomeric unit. This was indicated in the kinetic experiments by the linear relationship obtained with increasing protein concentrations up to 1 mg/ml. Above this concentration, further experimentation would be required to establish whether there is any departure from monomeric behaviour.

The small size of chorismate lyase is not surprising, since more and more proteins are being isolated of similar size, which are nonetheless very efficient at carrying out their catalytic function. It is interesting to compare chorismate lyase with ribonuclease of molecular weight 12,000 and lysozyme of molecular weight 13,900.

Both these proteins are small, but they do not lack efficiency or specificity in their catalytic function.

Considering the regulation of enzymic reactions, it is often found that the end-product of a pathway regulates the first enzyme of the sequence. Where branched pathways are concerned, the 'branch-point' enzyme is usually repressed and this results in the pathway ceasing to function. With regard to chorismate lyase activity and the ubiquinone pathway, studies on the regulation of chorismate lyase indicate that ubiquinone does not regulate the level of chorismate lyase. It was considered possible that other intermediates in the pathway could be responsible for repressing the activity of the enzyme, and initial studies have shown that 2-octaprenylphenol or an earlier biosynthetic intermediate could be involved with the repression of the enzyme. Results from kinetic studies on chorismate lyase indicate that it is inhibited very strongly by the product of the first specific reaction, 4-hydroxybenzoate. Because of this inhibition, the first reaction after the branch point compound, chorismate, ceases to operate well before the concentration of 4-hydroxybenzoate reaches 50 μM.

The turnover number for chorismate lyase is .06 which is extremely low, as would be expected for a reaction in a minor pathway. This would indicate that the concentration of 4-hydroxybenzoate in the cell would probably never reach 50 μM, since the rate of enzymic conversion is

extremely low. It is important that rate of production of chorismate be sufficiently high at all times, as it is a branch-point compound, and is also required for the other major biosynthetic pathways. However, provided that the enzymic reactions in the pathway are well regulated, then the rate of production of any compound will be equal to its rate of removal.

Another approach to the regulation of chorismate lyase activity may possibly be connected with the second specific reaction in ubiquinone biosynthesis. This involves the enzyme 4-hydroxybenzoate octaprenyltransferase. The side chain precursor and the enzyme in this reaction are membrane bound (Young et al. 1972), unlike chorismate lyase which is soluble. However, a loose arrangement, or aggregate, could exist between chorismate lyase and 4-hydroxybenzoate octaprenyltransferase. This idea is based on the location of the two genes on the *E. coli* chromosome. Both the *ubiC* and *ubiA* genes map at approximately the same position and the formation of a gene cluster (Section 2.1) may be advantageous to the cell.

If a gene cluster for these reactions were to exist, one of the important advantages to the cell would be that an end-product of a chain of reactions could repress the synthesis of the two enzymes in the pathway. Secondly, if the gene products were extremely unstable their concurrent synthesis might require that the genes be very

close together. Thirdly, if the genes were close together this would then enable the metabolic products of the reactions to be handled with ease.

If an organisation, such as the above, were to exist in the cell, regulation of enzymic reactions would be much more efficient. For example, a high concentration of substrate in the immediate vicinity of the enzyme would not be required. Other complexes have been shown to occur in the aromatic pathway and one that occurs in the common pathway of N. crassa consists of five enzymes (Giles et al. 1967). This complex catalyses the reactions from 3-deoxy-D-arabino-heptulosonic acid 7-phosphate to 3-enolpyruvyl shikimate-5-phosphate (Figure 1.1). Burgoyne et al. (1969) have purified this arom complex and estimated it to have a molecular weight of 231,000.

During the isolation procedure there appeared to be no separation of the five enzymic activities. However, when the complex was dissociated into lower molecular weight species, a loss in all of the activities was observed. The possible function of this arom gene cluster (or any other gene cluster) has been suggested by Giles et al. (1967) to facilitate the aggregation of biosynthetic enzymes and as well to separate the synthetic from the degradative pathway in cases where the pathways are competitive. This could very well apply to biosynthetic pathways where a single compound, such as chorismate, could be channelled in numerous directions. Other

complexes of enzymes have also been shown to occur in aromatic biosynthesis, e.g. Nester et al. (1967) have shown that in B. subtilis, DAHP synthetase, shikimate kinase and chorismate mutase are physically associated. Another aggregate in N. crassa involved with tryptophan synthesis has been shown to contain three enzymes which behave as one homogeneous protein (Gaertner and DeMoss, 1969). This involves the enzymes anthranilate synthetase, N-(5'-phosphoribosyl)-anthranilate isomerase and glycerol phosphate synthetase.

The presence of so many aggregates in aromatic biosynthesis favours the likelihood of chorismate lyase and 4-hydroxybenzoate octaprenyltransferase in E. coli being able to form an aggregate. However, a detailed study on the biochemical properties of these two enzymes would be required to establish aggregate formation.

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